Biology and Physiology of Freshwater Neotropical Fish

Edited by
Bernardo Baldisserotto
Elisabeth Criscuolo Urbinati
J.E.P. Cyrino

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Chapter 1

Phylogeny and classification of Neotropical fish

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Chapter outline

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Phylogenies and organization of biological diversity 2
Phylogenetic relationships between groups of modern fish and the Tetrapodomorpha 3
Diversification of Actinopterygii and main Neotropical lineages 5
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Introduction

Actinopterygian fish are the richest group among vertebrates, corresponding to approximately half the number of species of animals with backbones. Contrastingly, knowledge about the ichthyofauna diversity in the world is still far from complete. Through early 2018, more than 34,700 fish species were recognized, but this number is constantly increasing due to the high rate of description of new taxa. In the last decade (2008–17), an average of 420 fish species were described per year, and just in 2017, 469 new species were added (Eschmeyer et al., 2018).

The freshwaters of the Neotropical region (South and Central Americas) include 20%–25% of all world fish diversity, currently including more than 6000 species with final estimates varying between 8000 and 9000 species (Reis et al., 2016). Amazingly, this huge diversity is concentrated in less than 0.003% of the available water resources on the planet, corresponding to the Neotropical freshwaters (Vari and Malabarba, 1998). Most of the fish in this region belong to a single large group of primary freshwater fish (Box 1.1): the Otophysa. Otophysans became dominant in the occupation of the planet's freshwater environments and constitute about 72% of Neotropical freshwater fish species, being represented in South and Central America by the Characiformes, Siluriformes, and Gymnotiformes (Fig. 1.1). Besides the Otophysa, Neotropical fish consist of two important groups of secondary freshwater fish (Box 1.1): Cyprinodontiformes (about 13%) and Cichliformes (about 9%). The remaining 6% are made up of 23 different orders, mostly peripheral fish of marine origin.

BOX 1.1

The ecophysiological classification in primary, secondary, and peripheral freshwater fish refers to the origin of the groups and their tolerance to salinity (Myers, 1949, 1966). Primary freshwater fish groups are those originated in freshwater and intolerant to salt water, such as electric eels (Gymnotiformes) and tetras and relatives (Characiformes). Secondary freshwater fish groups are those salt water tolerant, such as cichlids (Cichlidae) and poeciliids (Cyprinodontiformes). Peripheral freshwater fish groups are those nondiadromous freshwater species belonging to groups of marine origin with freshwater representatives, such as freshwater stingrays (Potamotrygonidae), silversides (Atheriniformes), gobies (Gobiiformes), and anchovies (Clupeiformes).

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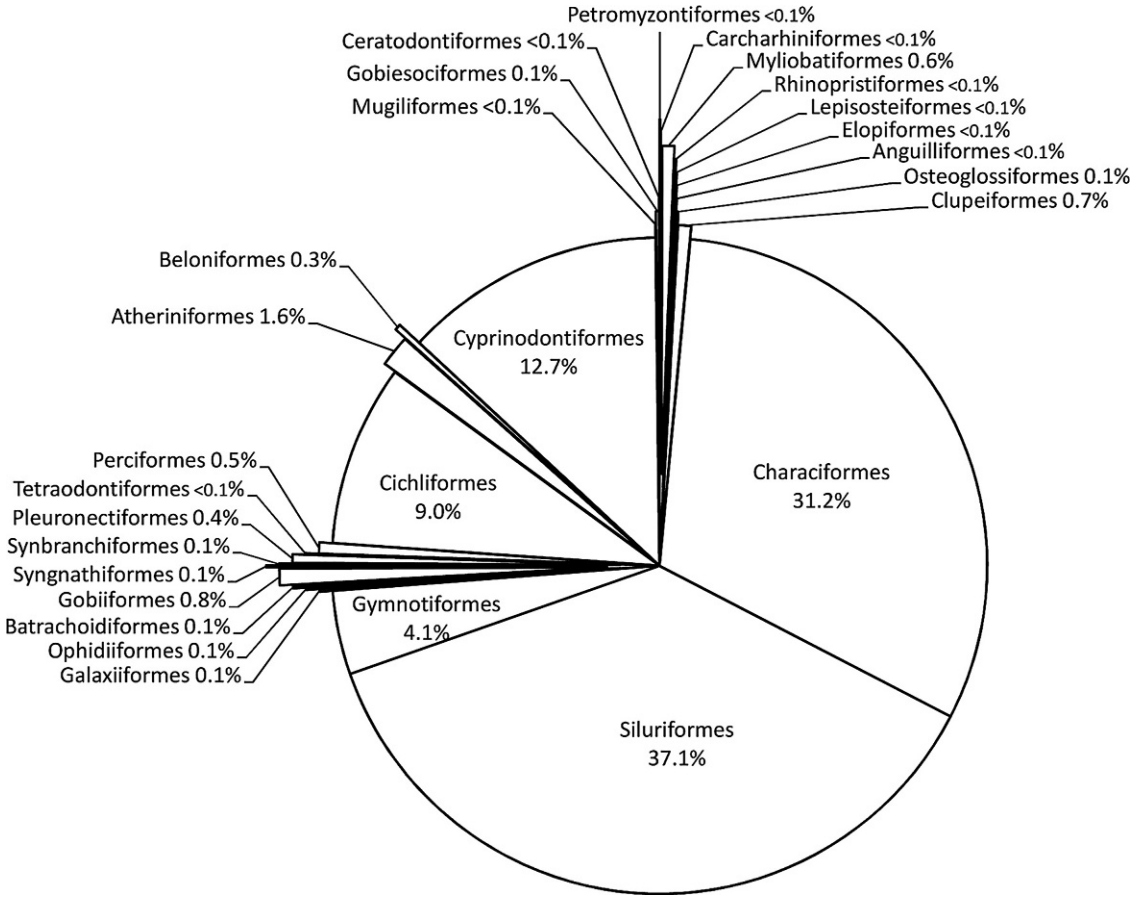

FIG. 1.1 Percentage of each fish order in the composition of the Neotropical fish fauna. Total number of species (6323) and number of species per order inhabiting Neotropical freshwaters updated July 2018 by checking Eschmeyer et al. (2018) list of fish species. Note that the Otophysa (represented by Characiformes, Siluriformes, and Gymnotiformes), along with the Cypriniformes and Cichliformes, constitute about 94% of this fauna.

Phylogenies and organization of biological diversity

Phylogenies aim to represent as accurately as possible the evolutionary history of organisms by defining natural (monophyletic) groups. All organisms belonging to a certain monophyletic group, also called a clade, have a single common ancestor, which is not an ancestor of any organism external to the group. These clades should be reflected in the taxonomic classification in monophyletic genera, families, orders, classes, phyla, and kingdoms. Smaller monophyletic groups (genera) are part of larger monophyletic groups (families) and so on.

In this way, a certain classification must be based on the evolutionary relationships among the taxa involved. The formulation of relationship hypotheses can be done by searching for characteristics that document these relationships. Any inheritable characteristic among individuals of a phylogenetic lineage, be it morphological, physiological, biological, behavioral, or molecular, is potentially informative for the reconstruction of the evolutionary history and the recognition of monophyletic groups. Characteristics that constitute a novelty in the evolution of a group (the appearance of the jaw in vertebrates, for example) allow us to formulate the hypothesis that all organisms that share this characteristic descend from a common ancestor, in which this characteristic arose for the first time. Thus, sharks are closer relatives of mammals than of lampreys, because they share a derived characteristic (presence of a jaw) that is absent in lampreys. Derived characteristics shared by more than one taxon allow us to formulate hypotheses of relations; these are called synapomorphies.

The formulation of a phylogenetic classification is the best way to organize information on biodiversity and has become a universal practice in biological classifications. The understanding not only of specific diversity but also of phylogenetic diversity provides us with a comparative view of the evolution and distribution of characteristics among lineages, be they

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morphological, biological, or molecular. Characteristics of any kind are distributed among living beings according to their phylogenetic origin, and as such serve as sources of information for the discovery of this phylogenetic history.

For example, ostariophysans have an unique alarm substance housed in holocrine cells in the epidermis (club cells). Their rupture causes a stereotyped reaction of fear between individuals of the same species, or even in other ostariophysans. This characteristic only occurs in Ostariophysi (including the orders Gonorynchiformes, Cypriniformes, Characiformes, and Siluriformes; lost secondarily in the electric fish of the order Gymnotiformes), evidencing the unique origin of the group (Fink and Fink, 1981). Thus, we can infer that such secretory cells are present in species of tetras (Characiformes) or catfish (Siluriformes), which were never investigated in relation to their epidermis. However, we should not expect their presence in cichlids, for example, that are not closely related to the Ostariophysi.

Another example concerns the organization of the testes during spermiogenesis. Fish of the superorder Atherinomorphae, including Atheriniformes (silversides), Cyprinodontiformes (killifish), and Beloniformes (needlefish), have a unique pattern of spermiogenesis with the spermatogonia forming at the distal end of each lobe of the testis and the other stages of spermiogenesis succeeding toward the lumen. In the other teleosts, the spermatogonia form along the entire tubule, interspersed with other phases of spermiogenesis (see Grier et al., 1980: Fig. 1). Accordingly, we can suppose that this unique type of spermiogenesis arose in a common ancestor of all Atherinomorphae, and that occurs in the testes of other species of the group not yet investigated. We should not assume, however, that this type of spermiogenesis occurs in species of groups unrelated to Atherinomorphae, as in Characiformes, for example.

Phylogenetic relationships between groups of modern fish and the Tetrapodomorpha

Currently, the term "fish" refers to all aquatic vertebrates without terrestrial locomotive members, but it does not constitute a taxonomic group (Pisces), as it was widely used in the past. As Tetrapodomorpha emerged from sarcopterygian fish, when we exclude tetrapods from the fish clade, we make "fish" a paraphyletic group (includes an ancestor but not all of its descendants). Vertebrates we call "fish" include at least six distinct modern lineages with unique phylogenetic histories, and therefore, should be treated separately (Fig. 1.2): Mixini, Cephalaspidomorphi, Chondrichthyes, Coelacanthimorpha, Dipnomorpha, and Actinopterygii.

The Mixini, or hagfish, have been considered the sister group of all vertebrates. They are exclusively marine, cold water, and antitropical. Cephalaspidomorphi, or lampreys, are freshwater or diadromous (see Box 1.2), in the latter case inhabiting

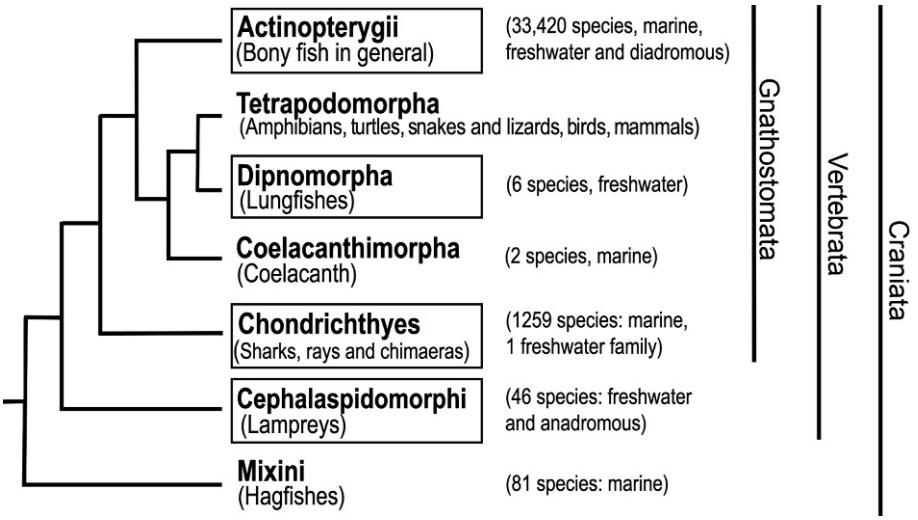

FIG. 1.2 Phylogeny of Craniata representing the relationship among the six lineages of modern fish and tetrapods. The number of species in each group (updated July 2018 according to Eschmeyer et al., 2018) and the habitat (marine, freshwater, or diadromous) are informed on the right. The four groups included in boxes have representatives in the Neotropical Ichthyofauna.

BOX 1.2

Fish that migrate between freshwater and salt water to spawn are called Diadromous. Those that enter freshwater to spawn and migrate to the ocean as juveniles to grow into adults are termed Anadromous (e.g., *Genidens barbus*, Ariidae). Fish that spawn in salt water and migrate to freshwater to grow into adults are termed Catadromous (e.g., *Mugil liza*).

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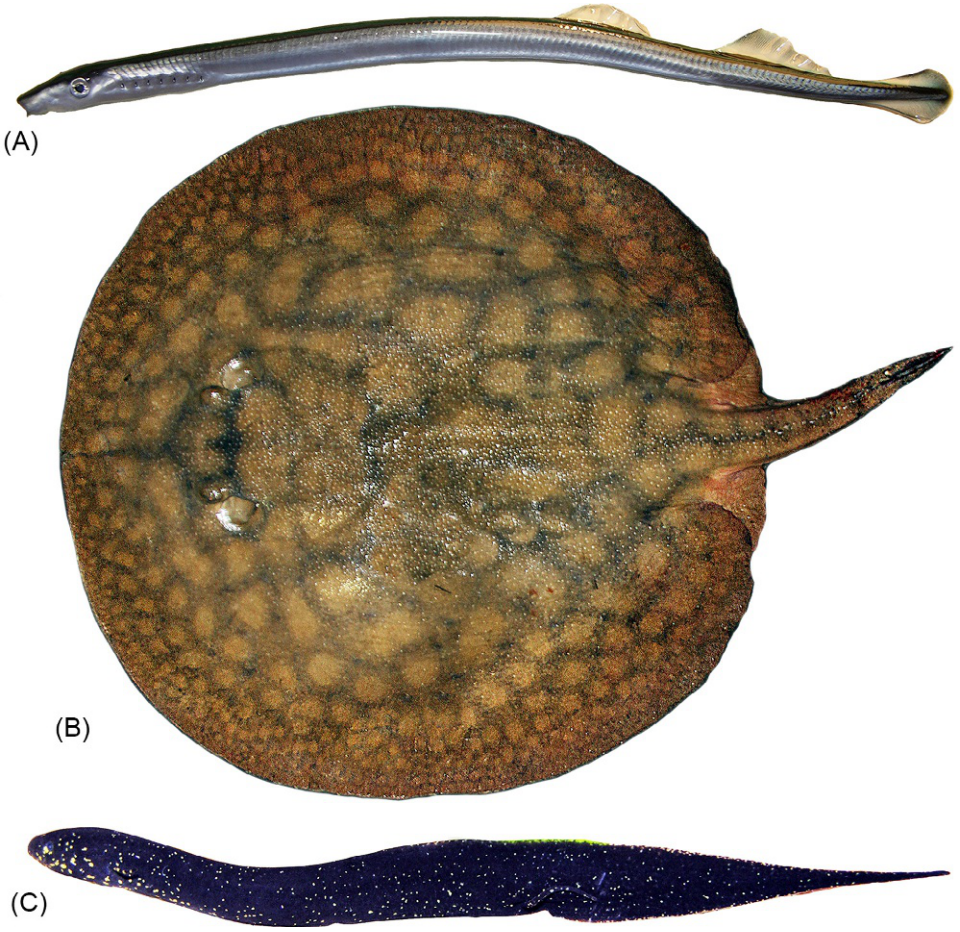

FIG. 1.3 Some representatives of the (A) Cephalaspidomorphi (lampreys), (B) freshwater Chondrichthyes (Potamotrygonidae ray), and (C) Dipnomorpha (lungfish).

the marine environment in the adult phase and returning to freshwater for spawning and larval development (anadromous). In South America, only two species of lamprey occur in the southern hemisphere continent, *Mordacia lapicida* (Gray, 1851), endemic to Chile, and *Geotria australis* Gray, 1851, present in Chile, Argentina, Australia, and New Zealand (Malabarba and Malabarba, 2007). Hagfish (Mixini) and lampreys (Cephalaspidomorphi) do not have a jaw, which easily differentiates them from other vertebrates (Fig. 1.3A).

Chondrichthyes are marine with a family of freshwater stingrays, Potamotrygonidae (Fig. 1.3B), which includes 37 species and is endemic to the large hydrographic basins of South America. The monophyly of the family supports the hypothesis that all Neotropical freshwater stingrays descend from one ancestral species that invaded this environment from a marine ancestor (Lovejoy, 1996), being considered as a group of peripheral freshwater fish. Potamotrygonidae are phylogenetically related to the Dasyatidae family of marine stingrays, having as their sister group two stingrays of the genus *Styracura*, *S. schmardae* (Werner, 1904), and *S. pacifica* (Beebe and Tee-Van, 1941), which occur on the Caribbean coast of South America and the Pacific coast of Central America, respectively (Lovejoy et al., 2006). In addition to Potamotrygonidae, the flat-headed shark *Carcharhinus leucas* (Valenciennes, 1839) of the Carcharhinidae family, and two species of sawfish (Pristidae), *Pristis pectinata* Latham, 1794 and *Pristis pristis* (Linnaeus, 1758), occur in freshwater environments in the northern region of South America and in Central America.

Coelacanthimorpha (=Actinistia) and Dipnomorpha correspond to lineages more related to Tetrapodomorpha than to other fish. Characteristics evidencing these relationships include the fin skeleton, which has homologous bones (of the same origin) to the bones of the locomotor members of the tetrapods (such as humerus, radius, and ulna, for example). Dipnoans (lungfish) also present a lung for air breathing, homologous to the lung of the Tetrapodomorpha. Coelacanthimorpha, although abundant in the fossil record, has only two modern species. They are both marine and considered living fossils, occurring at great depths on the east coast of Africa and Indonesia. Dipnomorpha is an exclusive freshwater group with only six modern species of lungfish, one in South America, four in Africa and one in Australia. The only species of lungfish in South America is the "piramboia," *Lepidosiren paradoxa* Fitzinger, 1837 (Family Lepidosirenidae, Fig. 1.3C).

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Actinopterygii are ray-finned fish (actin = radius, ptero = wing), which make up the vast majority of fish known today, both at sea and in continental waters. They have successfully assumed a great variety of sizes, forms, and habits, as adaptations for occupation of a wide range of aquatic environments. Thus, they are found from abyssal depths in the oceans to altitudes of more than 3821 m, as in Lake Titicaca, Peru, and Bolivia. They also occur both in perennial water environments and those with temporary waters, which dry during certain periods of the year. The group includes such diverse forms that, even though it is recognized as monophyletic, there is no set of strong synapomorphies defining it (Patterson, 1982; Nelson, 2006; Nelson et al., 2016). This is because some of the unique characteristics of the actinopterygians have changed during the evolution and diversification of the group, being modified or lost in more specialized forms, such as the histological structure of the scales, the acrodine hood in the teeth, the pelvic fin with an expanded metapterygium supporting radials, and fins supported by lepidotrichia (Lauder and Liem, 1983).

Diversification of Actinopterygii and main Neotropical lineages

The Actinopterygii are divided into two classes: Cladistia (bichirs), represented by a single modern order (Polypteriformes) restricted to Africa, and Actinopteri, with the subclasses Chondrostei and Neopterygii. Only the subclass Neopterygii occurs in South and Central America, branched in two infraclasses, Holostei and Teleostei. The Holostei (gars) are secondary freshwater fish with only eight modern species, three of the genus *Atractosteus*, family Lepisosteidae, occurring in Central America. There are four major groups within Teleostei: Elopomorpha, Osteoglossomorpha, Otomorpha, and Euteleosteomorpha (Fig. 1.4).

Elopomorpha are primarily marine fish, including the tarpon and eels. Among the synapomorphies that attest to the monophyly of the group, the stage of leptocephalous larvae in development is certainly the most interesting. As a ribbon and commonly 10 cm long, but reaching up to 2 m, the larva shrinks during metamorphosis into the juvenile form. Only three species of Elopomorpha are known to occur in the freshwaters of the Neotropical region. The tarpon, *Megalops atlanticus* Valenciennes, 1847 (Megalopidae), is marine and found at the mouths of rivers and estuaries. The freshwater Eel, *Anguilla rostrata* (Lesueur, 1817) (Anguillidae), is found in rivers in the Caribbean region. This species spends most of the life cycle in freshwater, returning to the sea for spawning (catadrome). Finally, the snake eel, *Stictorhinus potamius* (Böhlke and McCosker, 1975) (Ophichthyidae), is unique to freshwater and endemic to the South American continent, occurring in the Orinoco, Amazonas, Tocantins, and northeast Brazilian basins.

Osteoglossomorpha (bonytongues) is a relatively small group of freshwater teleosts and yet morphologically diverse, especially on the African continent. In South America, they are represented by two families of fish popularly known as arowanas (Osteoglossidae, two species, Fig. 1.5A) and pirarucus (Arapaimidae, possibly four species), being *Arapaima gigas* (Schinz, 1822), the largest species of freshwater fish in the Neotropics. They have a basal position among teleosts and are sometimes referred to as the living brother group of all others. Recent phylogenetic studies (Wiley and Johnson, 2010) list several synapomorphies for the group, among which are the absence of epipleural bones, one epural, preural vertebra one with complete neural spine, and supraorbital absent.

Until recently, Clupei (=Clupeomorpha) and Ostariophysi were distinct groups in Actinopterygii, but currently they are classified as two subcohorts of the Otomorpha (Fig. 1.6). Three synapomorphies define the group (Arratia, 1999): the medial extrascapular fused to the parietal or fused to the parietal and supraoccipital; autopalatine ossifying early on ontogeny; and bases of hipural 1 and 2 not united by cartilage at any stage of growth. Clupeomorpha includes the Clupeiformes, and Ostariophysi includes the Gonorynchiformes and the Otophysa with four orders: Cypriniformes, Characiformes, Siluriformes, and

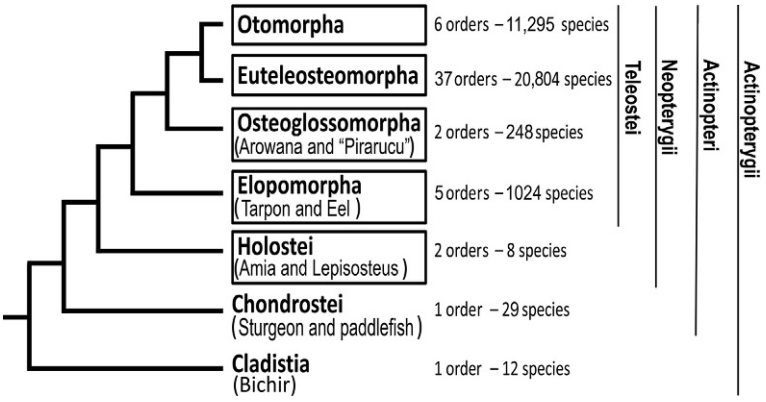

FIG. 1.4 Phylogeny of Actinopterygii, detailing the relationship of the classes Cladistia and Actinopteri, the subclasses Chondrostei and Neopterygii, the infraclasses Holostei and Teleostei, and the cohorts Elopomorpha, Euteleosteomorpha, Otomorpha, and Osteoglossomorpha. The five groups included in boxes have representatives in Neotropical Ichthyofauna. The number of orders and the total number of species in each group (updated July 2018 according to Eschmeyer et al., 2018) are informed on the right.

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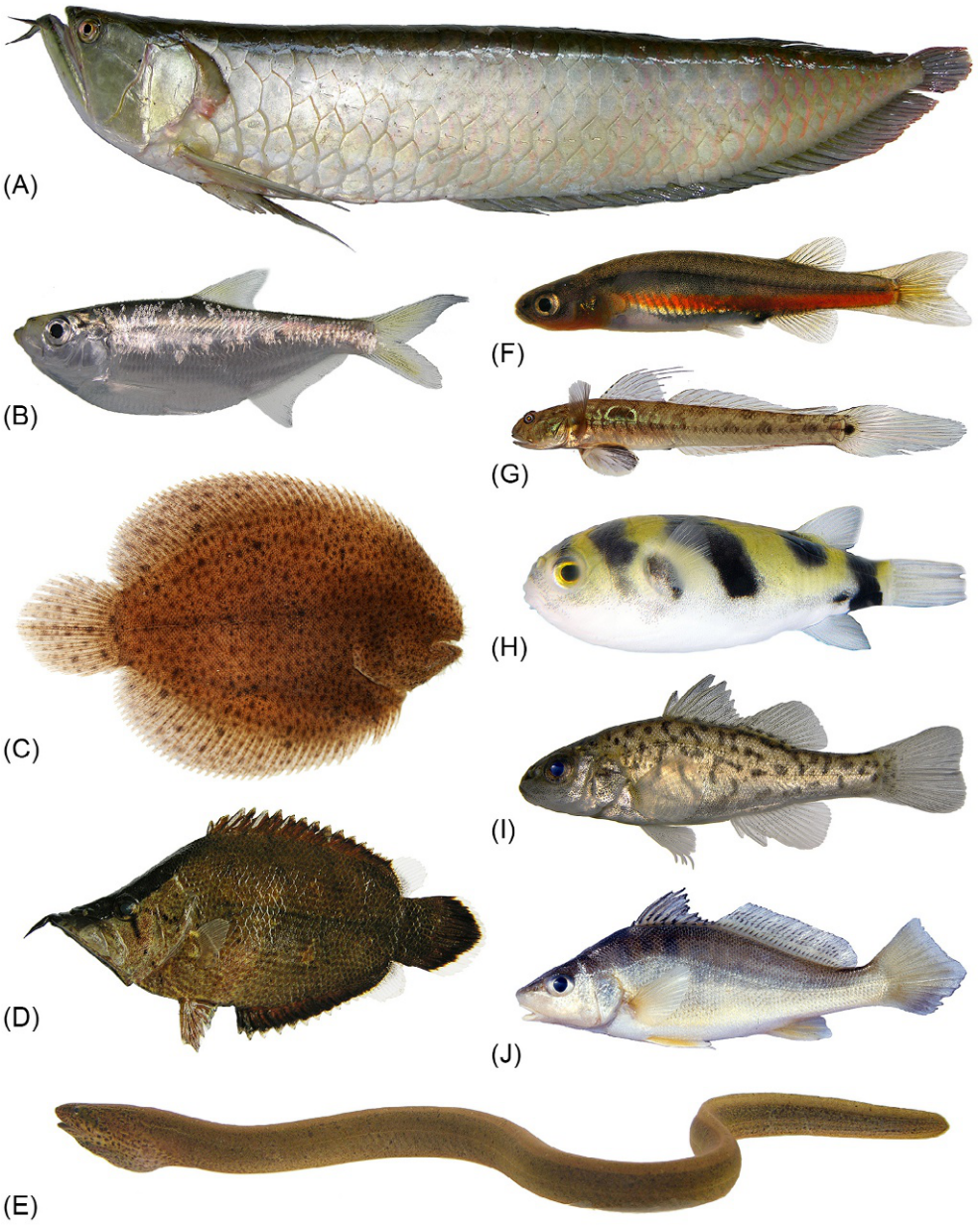

FIG. 1.5 Some representatives of (A) Osteoglossiformes, (B) Clupeiformes, (C) Pleuronectiformes, (D) Ovalentaria (Polycentridae), (E) Synbranchiformes, (F) Galaxiiformes, (G) Gobiiformes, (H) Tetraodontiformes, two Perciformes (I) Perciliidae, and (J) Scianidae. Not in scale.

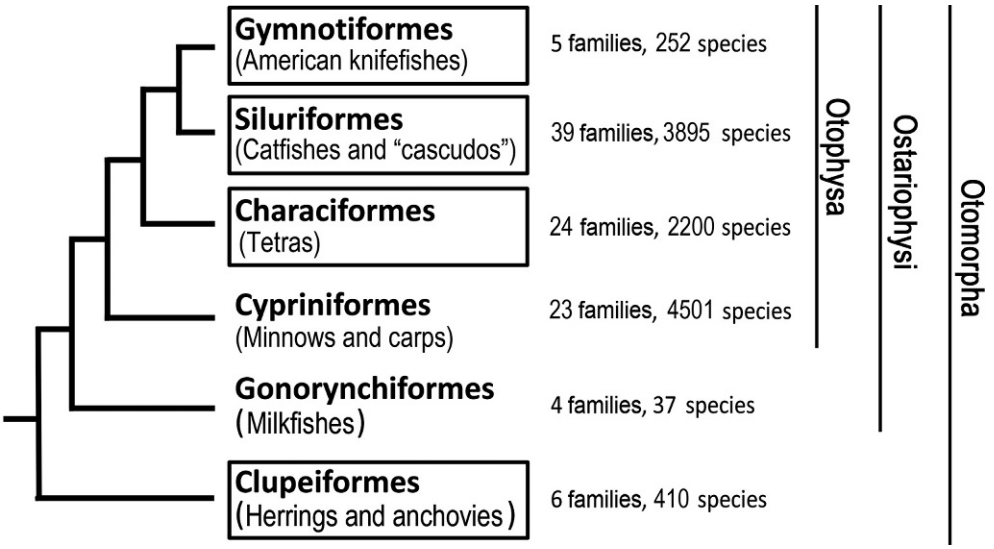

FIG. 1.6 Phylogeny of Otomorpha. The four orders represented in boxes have representatives in Neotropical Ichthyofauna. The number of species in each group updated July 2018 according to Eschmeyer et al. (2018).

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Gymnotiformes. The Alepocephali was tentatively included as a third subcohort of the Otomorpha based on molecular studies, but so far no morphological synapomorphies have been found to support this hypothesis (Betancur et al., 2017).

Clupeiformes (herrings and anchovies) are mostly marine with many estuarine or diadromous species, and some fully adapted to life in freshwater (Fig. 1.5B). The freshwater species range in size from a few centimeters, such as *Platanichthys platana* (Regan, 1917), common in coastal lagoons and lower portion of rivers of southern Brazil, Uruguay, and Argentina, up to about 50 cm length measured to the base of the caudal fin, as *Pellona castelnaeana* (Valenciennes, 1847) of the Amazon river basin. Two synapomorphies support the group: the presence of abdominal keels in the ventral midline of the body and the presence of a connection between the swim bladder and the inner ear, with a swimming bladder diverticulum that penetrates the exoccipital and expands to form an ossified bula on prootic, and sometimes on pterotic.

Ostariophysi is a group of primarily freshwater fish of global distribution. The monophyly of the Ostariophysi and its inner groups is supported by a series of synapomorphies described in Fink and Fink (1981, 1996). Many of these synapomorphies are related to modifications of the inner ear, swimming bladder, and the four or five anterior vertebrae, which form a chain of ossicles that connects the swim bladder to the inner ear, the Weber apparatus (Fig. 1.7; Box 1.3). Another relevant synapomorphy in the group is the presence of an alarm substance in the epidermis, secondarily lost in

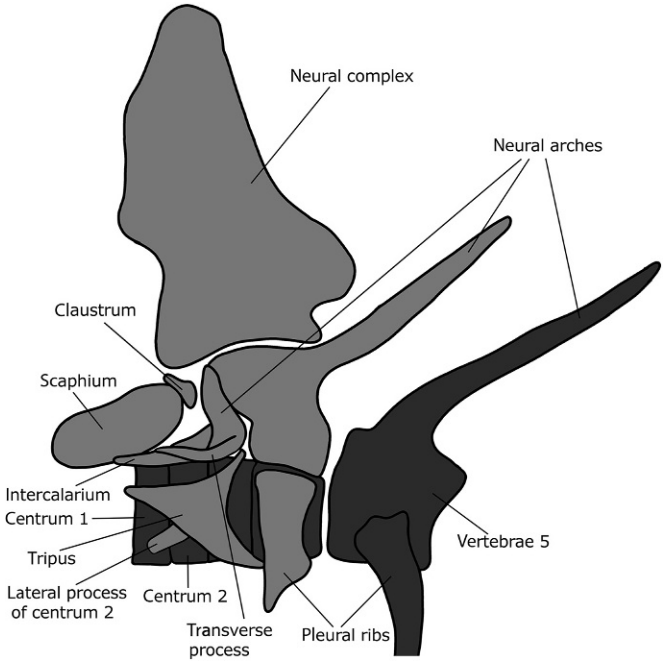

FIG. 1.7 Representation of the anterior vertebral region in a generalized characiform showing the Weberian apparatus.

BOX 1.3

The Ostariophysi comprises one of the largest and most diversified groups of teleosts. Despite their remarkable diversification and specializations, all ostariophysans, and uniquely them (Fink and Fink, 1981, 1996), present a complex bony structure connecting the inner ear to the swimbladder, which is called the Weberian apparatus. The Weberian apparatus was first described by Weber (1820), for whom it is named, and represents an important step in understanding the evolution of hearing in ostariophysan fish. The structure is basically made up of a series of modifications on anterior vertebrae and supraneurals making possible the sound transmission from the swimbladder to the inner ear (Rosen and Greenwood, 1970; Grande and Young, 2004). When the gasbladder pulsates in a sound field, high-frequency vibrations are transmitted from it via the Weberian ossicles to the inner ear, inside the skull (Alexander, 1962, 1964). This system, along with the lateral line system, enables these fish to receive a wide range of sound sources (Ladich, 1999; Grande and Young, 2004). Although there is still debate about which vertebral component originated each of the elements in the apparatus (Grande and Young, 2004), it is undeniable that the Weberian apparatus is the most remarkable of the ostariophysan synapomorphies.

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Gymnotiformes in which the sensorial stimulus is mainly electric. Gonorynchiformes (milkfish) include 2 marine species and 35 freshwater African species. The Cypriniformes (carps and minnows), with more than 4500 species, are the dominant group in freshwater in the Northern Hemisphere and with great representativeness in Africa.

Diversity and classification of Neotropical Otophysa

The Characiformes

Characiformes is one of the main orders of Ostariophysi, with more than 2200 species (Fig. 1.8). They occur only in freshwater, in Africa and South, Central, and North America as far as Texas. Although they occur on both sides of the Atlantic,

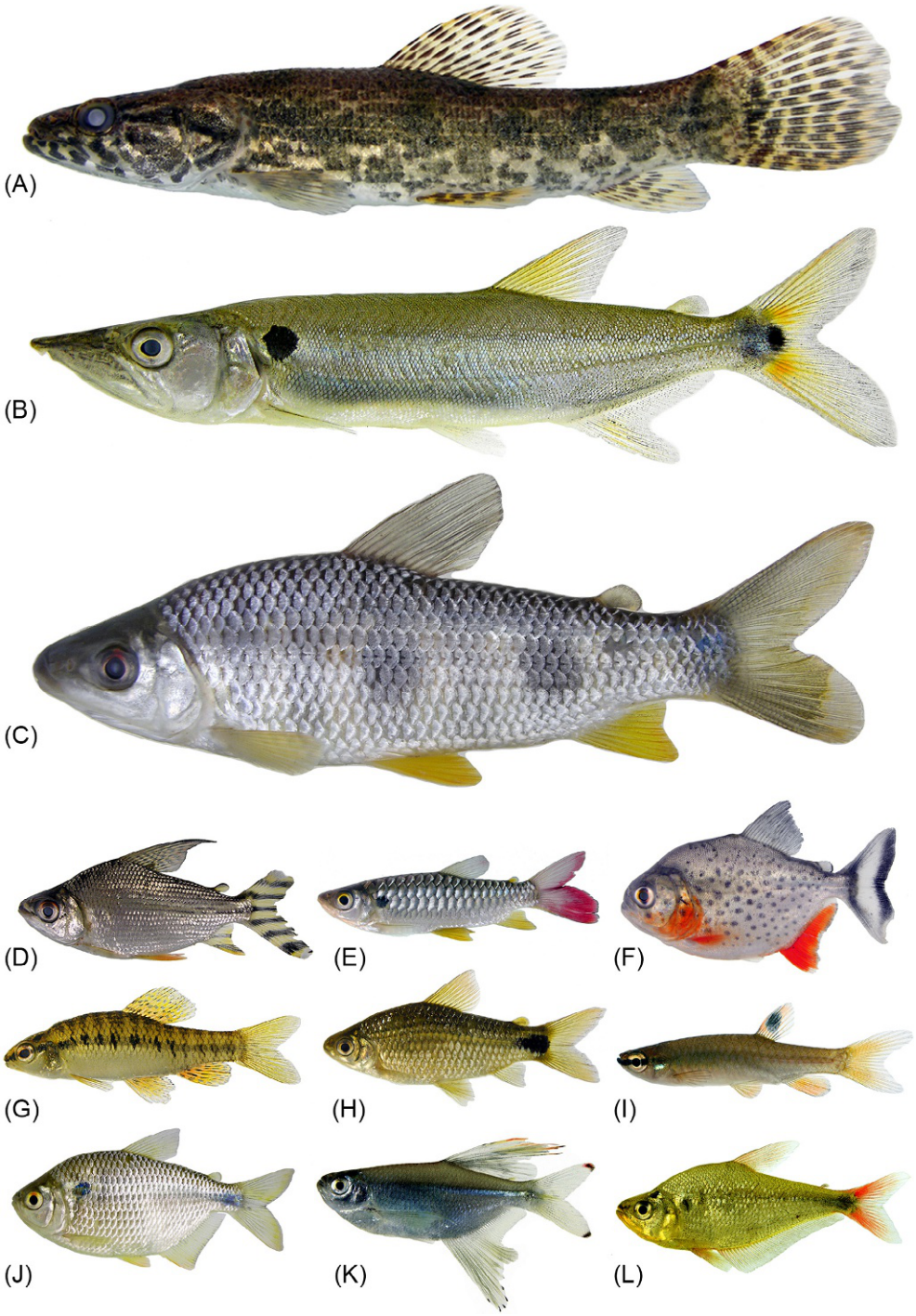

FIG. 1.8 Some representatives of Characiformes: (A) Erythrinidae, (B) Acestrorhynchidae, (C) Anostomidae, (D) Prochilodontidae, (E) Chalceidae, (F) Serrasalmidae, (G) Crenuchidae, (H) Curimatidae, (I) Lebiasinidae, and three members of the Family Characidae: (J) *Astyanax*, (K) *Pseudocorynopoma*, and (L) *Charax*. Not in scale.

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their diversity and richness is much greater in the Neotropical region, where there are 234 genera versus only 38 genera in Africa (Malabarba and Malabarba, 2010). Its current distribution and fossil records document the historical connection between Africa and South America. The order was defined by Fink and Fink (1981, 1996) based on seven synapomorphies, highlighting the presence of a lagenar capsule, replacement for outer-row dentary teeth and some premaxillary teeth formed in crypts, and the presence of multicuspid teeth. However, the phylogenetic relationships between the families are still complex, with no consensus about the relationships among their components.

Characidae, the richest family of the order with almost 1200 species, has been recently defined to include small size species (Fig. 1.8J–L) characterized by the absence of supraorbital bone (Malabarba and Weitzman, 2003; Azevedo, 2010; Oliveira et al., 2011). Although the Characiformes are fish of external fertilization, in at least three groups of characids occurs an alternative strategy of reproduction denominated of insemination, in which males transfer the spermatozoa to the female ovary through a ritual involving complex cohort behavior. Fertilization, however, does not occur in the ovaries, and the exact moment of the oocyte fertilization is still unknown. This strategy apparently has some adaptive advantages, such as separating the cohort and spawning moments, allowing the female to spawn regardless of the presence of the male whenever environmental conditions are favorable. Characids have been shown to have a high variability in sperm shape and ultrastructure, especially in inseminating species, presenting modified spermatozoa in their shape and distribution of organelles, whose analysis has provided a number of new characteristics in phylogenetic studies (Burns et al., 2009; Quágio-Grassiotto et al., 2012).

A second major group of Characiformes has been referred to as the superfamily Anostomoidea. It includes the families Chilodontidae and Anostomidae (Fig. 1.8C) that show a markedly diverse skull morphology and trophic ecology, and eat primarily a variety of plants and invertebrates; and the families Curimatidae (Fig. 1.8H) and Prochilodontidae (Fig. 1.8D) that are detritivorous (Sidlauskas, 2008). Because of their large number and annual migrations, these species are exploited in commercial and subsistence fisheries as important food items in South America. Recent phylogenetic studies have placed the Parodontidae as a sister group to the clade formed by these four families (Arcila et al., 2017).

Serrasalmidae are deep-bodied Characiformes (Fig. 1.8F) characterized by the presence of a series of midventral abdominal spines. The carnivorous members of the family (piranhas) possess one row of interlocking, sharp, and pointed teeth on each jaw that they use to cut fins or flesh from their preys. The herbivorous members (pacus) show molariform or incisiform teeth to grind fruits and seeds or to cut leaves. The large species of *Colossoma*, *Piaractus,* and *Mylossoma* are important species in commercial fishing and in farming (Jégu, 2003).

The Crenuchidae is a species-rich group of small characiform fish (Fig. 1.8G), usually reaching less than 10 cm. They are diagnosed by the presence of paired foramina located in the frontal bones, posterodorsally to the orbits (Buckup, 2003). Most species of Crenuchidae are inhabitants of fast flowing small streams, where they hover around pebbles, rocks, and vegetation. Several species are miniature, reaching maturity below 25 mm SL. The Lebiasinidae is also a diverse group of small size Characiformes, ranging in size from 16.2 to 150 mm measured to the base of the caudal fin (Toledo-Piza et al., 2014). They are characterized by the elongate, cylindrical body shape (Fig. 1.8I) with large scales, a laterosensory canal system reduced or absent on the body, and an anal fin that is short with up to 13 rays.

Neotropical Characiformes also includes four not closely related families of medium- to large-size carnivorous fish with caniniform teeth, the Acestrorhynchidae (Fig. 1.8B), Ctenoluciidae, Cynodontidae, and Erythrinidae (Fig. 1.8A). The Gasteropelecidae and Triportheidae are characiforms usually with a large coracoid bone in the pelvic girdle and upturned mouths, adapted to feed near the surface. The Bryconidae include the largest American characiform, the dourado (*Salminus brasiliensis*) that reaches 1 m in length, along with the piraputangas (*Brycon* sp.), important in commercial fishing and farming. Other less speciose families are the Chalceidae (Fig. 1.8E), Hemiodontidae, and Iguanodectidae. The Tarumaniidae is the most recent discovery (de Pinna et al., 2017), representing the description of a new lineage of Characiformes with fossorial habits and great morphological divergence from the rest of the order. The only known species, *Tarumania walkerae*, is a predator with an 11-chambered swimbladder extending along most of the body. It inhabits leaf-litter deposits in the Rio Negro drainage, and is found deeply buried in isolated pools during the dry season, enlarging the array of life strategies found in Neotropical fish.

The Siluriformes

Siluriformes (catfish) is a group of freshwater fish except for the mainly marine Ariidae and Plotosidae families, but which also include representatives in brackish and freshwater. They occur on virtually every continent (except Australia). They are diagnosed by several synapomorphies listed in Fink and Fink (1981, 1996), among which we can highlight the absence of scales (Fig. 1.9), the presence of spines in the pectoral and dorsal fins, the parietal fused to the supraoccipital providing a rigid structure to the neurocranium, vertebrae 2–4 fused into a "complex centrum," and the third and fourth neural arcs

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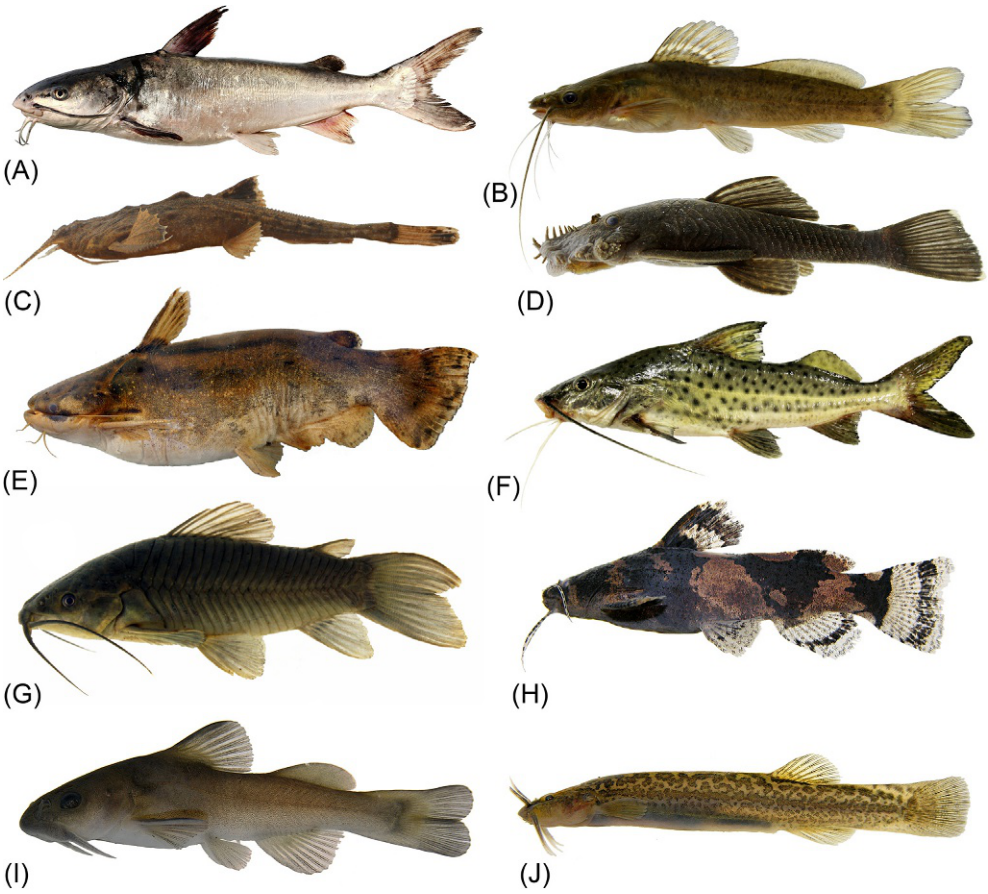

FIG. 1.9 Some representatives of Siluriformes: (A) Ariidae, (B) Heptapteridae, (C) Aspredinidae, (D) Loricariidae, (E) Auchenipteridae, (F) Pimelodidae, (G) Callichthyidae,(H) Pseudopimelodidae, (I) Diplomystidae, and (J) Trichomycteridae. Not in scale.

fused together and to the complex centrum. Even though it is a recognized monophyletic and intensely studied group, there are still several questions to be answered about classification and relationship between families.

Several siluriform species cause painful wounds through their fin spines, which in some cases (as such in Ariidae, Callichthyidae, Doradidae, Heptapteridae, Pimelodidae, Pseudopimelodidae) may possess poison produced by cells of the covering epithelium (Wright, 2015). Siluriformes are important commercial items for aquariums, feeding, or even sport fishing in various regions.

Of the 38 families of this order, 15 occur exclusively in Central and South America, in addition to the Ariidae (Fig. 1.9A) family that is cosmopolitan, with more than 2300 neotropical species (Ferraris, 2007, updated based on Eschmeyer et al., 2018): Ariidae (151 species, 58 Neotropical), Aspredinidae (45 species), Astroblepidae (81), Auchenipteridae (123), Callichthyidae (220), Cetopsidae (43), Diplomystidae (7), Doradidae (95), Heptapteridae (218), Lacantuniidae (1), Loricariidae (974), Nematogenyidae (1), Pimelodidae (114), Pseudopimelodidae (50), Scoloplacidae (6), and Trichomycteridae (307). About 80% of this richness (1833 species) is included in only five of these families: Callichthyidae, Heptapteridae, Loricariidae, Pimelodidae and Trichomycteridae.

The Loricariidae (suckermouth armored catfish) constitute the largest Neotropical siluriform family (Fig. 1.9D). Distributed from Costa Rica to northern Argentina, the loricariids are mostly cisandines, but several species inhabit the western Andes in the northern portion of the continent. They are essentially freshwater with almost no tolerance to salinity, except for a few exceptions, such as *Ancistrus* and *Hypostomus*, which are sometimes found in slightly brackish waters. With their sucking mouths and scraping teeth, they are mainly illiophagous, but vegetable remains, small invertebrates, and even wood (without being known how they are digested) are also part of their diets (de Pinna, 1998). The great diversity is associated with low fecundity and parental care, a combination comparable to that of the African cichlid flocks. Despite the great diversity of the more than 950 species, the monophyly of the loricariids is legitimized by much evidence, among which: bifid teeth with asymmetrical cusps; ventral disc in the mesetmoid; body partially or totally covered by three or more rows of bony plates bearing odontodes; and pterotic fused to supracleithrum and expanded posteriorly (Schaefer, 1987; Armbruster, 2004).

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The second most specious family among Siluriformes is Trichomycteridae, the "candirus" (Fig. 1.9J). They occur in all Neotropical drainages from Costa Rica to the Patagonian region, including the west of the Andes. More than 300 species are divided into eight subfamilies: Copionodontinae, Glanapteryginae, Sarcoglanidinae, Stegophilinae, Tridentinae, Trichogeninae, Trichomycterinae, and Vandelliinae. Among the synapomorphies supporting the monophyly of Trichomycteridae, certainly the most conspicuous is represented by the structure of the opercular apparatus. The interopercular is modified in a compact structure with a large base that serves as support for odontodes. The opercular is also modified for bearing odontodes, but this characteristic is absent in the copionodontines, some glanapterygines (*Pygidianops*, *Typhlobelus* and *Glanapteryx*), and stegophilines (*Megalocentor* and *Apomatoceros*). While the majority of representatives, belonging to the subfamily Trichomycterinae, feed on generalized items as small invertebrates, the trichomycterids include some of the most notable feeding specializations. The Vandelliinae, for example, feed exclusively on blood from the gills of other fish, representing the only hematophagous gnatostomates besides some bats (de Pinna, 1998). Stegophilinae, in turn, feed on scales, mucus, or shreds of flesh. However, the most notorious vandelliines are the "candirus" (*Vandellia cirrhosa* Valenciennes, 1846), well known in the Amazon region for accidentally penetrating the urethra of humans and other mammals.

Heptapteridae include small- to medium-sized Siluriformes rarely exceeding 20 cm measured to the base of the caudal fin (Fig. 1.9B). They are endemic to the Neotropics, commonly found in small body waters from Central and South America, including a few troglobitic species. The family is diagnosed by five synapomorphies (Lundberg and McDade, 1986; Ferraris, 1988; Lundberg et al., 1991): posterior limb of fourth transverse process laterally expanded above the swimbladder and notched once to several times; neural spines of Weberian complex centrum joined by a straight-edged, horizontal, or sometimes sloping bony lamina; the process for insertion of levator operculi muscle on posterodorsal corner of hyomandibula is greatly expanded; quadrate with free dorsal margin and bifid shape, its posterior and anterior limbs articulate separately with hyomandibula and metapterygoid; and the presence of an anteriorly recurved process ("mesethmoid hook") drawn out from the ventrolateral corner of the mesethmoid. They can be externally recognized by a combination of characteristics that includes: skin naked; cutaneous laterosensory canals simple; nares separated and lacking barbels; three pairs of barbels (maxillary, inner, and outer mentals); adipose fin well developed; caudal fin deeply forked, emarginate, rounded, or lanceolate; gill membranes free, branchial openings not restricted; orbital rim free or not; first dorsal- and pectoral-fin rays varying from having pungent spines to completely flexible or mostly segmented (Bockmann and Guazzelli, 2003).

Another diversified family is Callichthyidae (tamboatá, *Corydoras*), whose members are easily recognizable by having the body covered by two rows of bony plates on each side (Fig. 1.9G). They are relatively small and mostly bottom feeders, occurring in a wide variety of habitats in the largest drainages in South America and Panama. In addition, all callichthyids are air breathing fish: the air is swallowed on the surface of the water, passes into the intestine where the gas exchange is made and is expelled by the anus (Jucá-Chagas and Boccardo, 2006). This combination of an antidesiccation body cover and aerial respiration gives callichthyids a great tolerance to stagnant and muddy water and makes them apt to "move" by land, between bodies of water (de Pinna, 1998). In addition to the typical dermal coverage, the Callichthyidae share several synapomorphies, including: lateral line reduction, absence of the lacrimal-antorbital bone, absence of premaxillary teeth in adults, and infraorbital series reduced to two elements.

Although Pimelodidae (long-whiskered catfish, jama, mandis, guinea fowl, sorubins) form a moderately diverse family with little more than 100 species, there is no unique external trait identifying them (Fig. 1.9F). However, they can be recognized by a combination of characteristics that includes: naked skin (no scales or plates), cranial roof never covered by musculature but by a thin skin, three pairs of barbels (maxillary, external, and internal mental), nostrils well separated and without barbs, well-developed adipose fin, and pungent or at least resistant dorsal and pectoral fin spines (Lundberg and Littmann, 2003). Most pimelodids are carnivorous or omnivorous, more active at night or at evening when their sensitive barbels play an important role in searching for food. Pimelodids inhabit a wide range of habitats in the Neotropics, reaching the maximum diversity in the Amazon region, where they are abundant components of the ichthyofauna. Sexual dimorphism is practically nonexistent, fertilization is external, and no parental care is known. The size is generally moderate from 20 to 80 cm, but there are extreme examples such as the "jau" (*Zungaro zungaro*) that can reach 140 cm and the "piraíba" (*Brachyplatystoma filamentosum*) that can exceed 3 m in length (Lundberg and Littmann, 2003).

The Gymnotiformes

Gymnotiformes (knifefish) are Neotropical electric fish easily recognized by the anguilliform body (Fig. 1.10), with loss of the dorsal, adipose, pelvic, and caudal fins (only Apteronotidae and *Electrophorus* maintain a caudal fin). Despite the anguilliform shape, its movement is mainly due to the undulation of the anal fin that extends along almost the whole length of the

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FIG. 1.10 Some representatives of Gymnotiformes: (A) Apteronotidae, (B) Gymnotidae, (C) Hypopomidae, (D) Rhamphichthyidae, and (E) Sternopygidae. Not in scale.

body. This arrangement of the anal fin and its supporting skeleton results in the displacement of the anus to a more anterior position, placed under the head in some species.

All species of the order use the electrogenic musculature to produce a continuous electric field. That permanent electrical discharge has sensorial and communication functions, presenting distinct frequencies and wavelengths among species. Adults of the Amazon "poraquê," *Electrophorus electricus* (Linnaeus 1766), can reach 2.5 m in length and are capable of producing electric shocks of up to 600 V to stun their prey or for defense (Campos-da-Paz, 2003).

Albert (2001) lists several synapomorphies for this order, among which we can highlight the presence of hypaxial electrical organs, changes associated with active electroreception at high frequencies, absence of taste buds in the extraoral integument, subcutaneous eyes, absence of alarm substances in the epidermis, and the impressive ability to regenerate axial structures of the body posterior to the coelomic cavity. The latter feature allows electric fish to regenerate any posterior portions of the body eventually eaten by other organisms, as long as they do not reach the abdominal region and internal organs. Gymnotiformes are divided into five families with satisfactorily resolved relationships (Fig. 1.10): Sternopygidae, Apteronotidae, Rhamphichthyidae, Hypopomidae, and Gymnotidae.

Diversity and classification of Neotropical Euteleosteomorpha

The cohort Euteleosteomorpha includes all the remaining teleost fish (Fig. 1.11), forming the sister group of Otomorpha. Three synapomorphies support the monophyly of this cohort: presence of pattern 2 of supraneurals, stegural with anterodorsal membranous growth, and median caudal cartilages (Johnson and Patterson, 1996; Wiley and Johnson, 2010). Euteleosteomorpha is divided into the subcohorts Lepidogalaxii, Protacanthopterygii, Stomiatii, and Neoteleostei.

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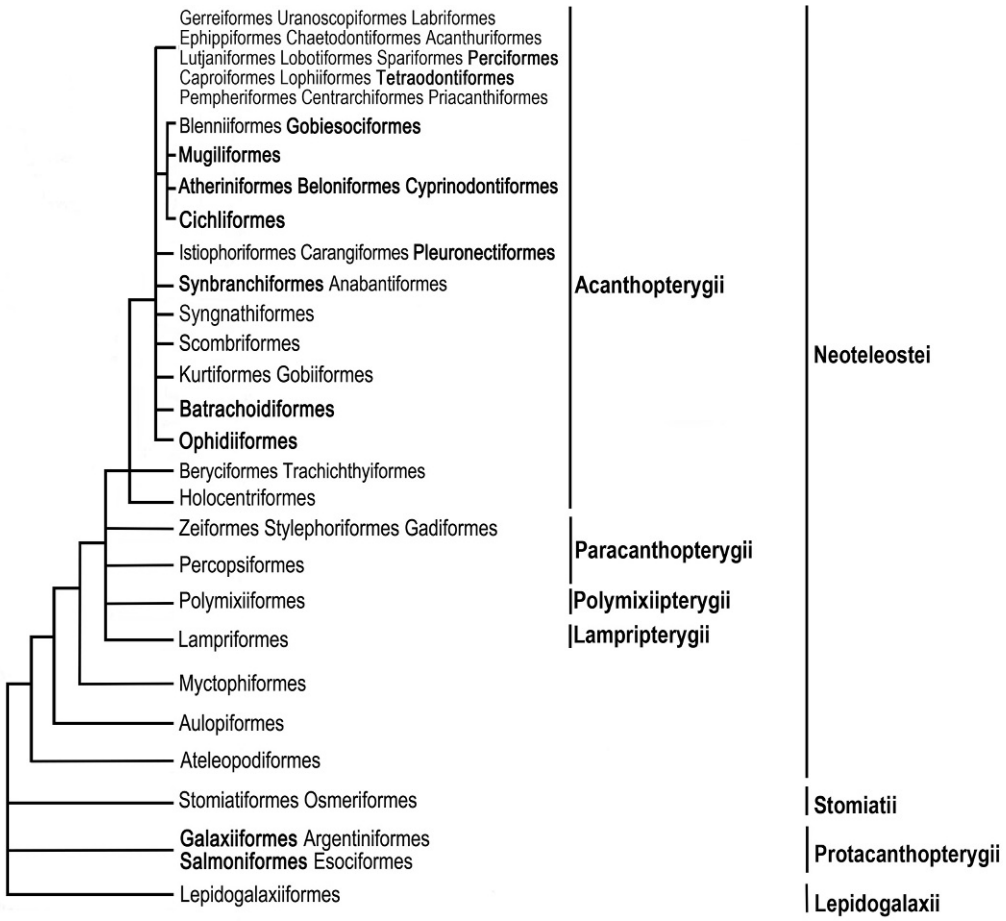

FIG. 1.11 Phylogeny of Euteleosteomorpha. Orders in bold have representatives in Neotropical Ichthyofauna.

The subcohort Lepidogalaxii contains a single species, *Lepidogalaxias salamandroides*, which survives dry seasons by burrowing into the mud. Protacanthopterygii is represented by the family Galaxiidae (Fig. 1.5F) in temperate cold waters in southern South America, with three genera and seven species of freshwater or diadromous fish (Malabarba and Malabarba, 2007). One of the species, *Galaxias maculatus*, has an impressive distribution for a freshwater species, occurring from Australia (including Tasmania) and New Zealand, to Argentina, Chile, and the Malvinas Islands. Such a distribution can only be explained by dispersal during the marine larval stage, which lasts up to 6 months. The subcohort Stomiati is divided into two orders: the Stomiatiformes, known as "dragonfish" that inhabit tropical to temperate deep sea waters, and the Osmeriformes, smelts that spawn in freshwater.

The subcohort Neoteleostei comprises two infracohorts (Ateleopodia and Eurypterygia), two sections (and Ctenosquamata), two subsections (Myctophata and Acanthomorphata), and four divisions (Fig. 1.5; Box 1.4). In addition to the five synapomorphies that define the group (Wiley and Johnson, 2010), most Neoteleostei presents the premaxillary with an ascending and articular process (Nelson, 2006), differently from the Protacanthopterygii.

Within division Acanthopterygii (=Euacanthomorphacea), the subdivision Percomorphaceae is the most diversified clade of the Euteleosteomorpha, dominating the oceanic waters and much of the tropical and subtropical continental waters. Several families of the Percomorphaceae have representatives in Neotropical freshwaters, including Achiridae (Fig. 1.5C), Batrachoididae, Bythitidae, Gobiidae (Fig. 1.5G), Gobiesocidae, Mugilidae, Percichthyidae, including Perciliidae (Fig. 1.5I), Polycentridae (Fig. 1.5D), Sciaenidae (Fig. 1.5J), Synbranchidae (Fig. 1.5E), Syngnathidae, and Tetraodontidae (Fig. 1.5H). The groups of Percomorphaceae with the greatest number of freshwater fish species comprise the superorders Atherinomorphae and Cichlomorphae, mainly the orders Cyprinodontiformes and Cichliformes, which are discussed below.

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BOX 1.4

Actinopterygii classification based on Betancur et al. (2017).

- Megaclass Osteichthyes (= extant Euteleostomi)
  - Superclass Actinopterygii
    - Class Cladistia (Order Polypteriformes)
    - Class Actinopteri
      - Subclass Chondrostei (Order Acipenseriformes)
      - Subclass Neopterygii
        - Infraclass Holostei (Orders Amiiformes and Lepisosteiformes)
        - Infraclass Teleostei
          - Megacohort Elopocephalai
            - Supercohort Elopocephala
              - Cohort Elopomorpha (Orders Elopiformes, Albuliformes, Notacanthiformes and Anguilliformes)
          - Megacohort Osteoglossocephalai
            - Supercohort Osteoglossocephala
              - Cohort Osteoglossomorpha (Orders Hiodontiformes and Osteoglossiformes)
            - Supercohort Clupeocephala
              - Cohort Otomorpha (= Otocephala, Ostarioclupeomorpha)
                - Subcohort Clupei (Order Clupeiformes)
                - Subcohort Alepocephali (Order Alepocephaliformes)
                - Subcohort Ostariophysi
                - Section Anotophysa (Order Gonorynchiformes)
                - Section Otophysa
                - Superorder Cypriniphysae (Order Cypriniformes)
                - Superorder Characiphysae (Order Characiformes)
                - Superorder Siluriphysae (Orders Siluriformes and Gymnotiformes)
              - Cohort Euteleosteomorpha
                - Subcohort Lepidogalaxii (Order Lepidogalaxiiformes)
                - Subcohort Protacanthopterygii (Orders Galaxiiformes, Argentiniformes, Salmoniformes and Esociformes)
                - Subcohort Stomiatii (Orders Stomiatiformes and Osmeriformes)
                - Subcohort Neoteleostei
                - Infracorte Ateleopodia (Order Ateleopodiformes)
                - Infracorte Eurypterygia
                - Section Cyclosquamata (Order Aulopiformes)
                - Section Ctenosquamata
                - Subsection Myctophata (Order Myctophiformes)
                - Subsection Acanthomorphata
                - Division Lampripterygii (Order Lampriformes)
                - Division Polymixiipterygii (Order Polymixiiformes)
                - Division Paracanthopterygii
                - Series Percopsaria (Order Percopsiformes)
                - Series Zeiogadaria
                - Subseries Zeiariae (Order Zeiformes)
                - Subseries Gadariae (Orders Stylephoriformes and Gadiformes)
                - Division Acanthopterygii (=Euacanthomorphacea)
                - Subdivision Berycimorphaceae (Orders Beryciformes and Trachichthyiformes)
                - Subdivision Holocentrimorphaceae (Order Holocentriformes)
                - Subdivision Percomorphaceae
                - Series Ophidiaria (Order Ophidiiformes)
                - Series Batrachoidaria (Order Batrachoidiformes)
                - Series Gobiaria (Orders Kurtiformes and Gobiiformes)
                - Series Pelagiaria (Order Scombriformes)
                - Series Syngnatharia (Order Syngnathiformes)
                - Series Anabantaria (Orders Synbranchiformes and Anabantiformes)
                - Series Carangaria (Orders Istiophoriformes, Carangiformes and Pleuronectiformes) (Order-level incertae sedis Centropomidae, Lactariidae, Leptobramidae, Menidae, Polynemidae, Sphyraenidae, Toxotidae)
                - Series Eupercaria (Orders Gerreiformes, Uranoscopiformes, Labriformes, Ephippiformes, Chaetodontiformes, Acanthuriformes, Lutjaniformes, Lobotiformes, Spariformes,

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BOX 1.4 —cont'd

Priacanthiformes, Caproiformes, Lophiiformes, Tetraodontiformes, Pempheriformes, Centrarchiformes, and Perciformes)
Series Ovalentaria (Order-level *incertae sedis* Ambassidae, Congrogadidae, Embiotocidae, Grammatidae, Opistognathidae, Plesiopidae, Polycentridae, Pomacentridae, Pseudochromidae)
Superorder Cichlomorphae (Order Cichliformes)
Superorder Atherinomorphae (Orders Atheriniformes, Beloniformes and Cyprinodontiformes)
Superorder Mugilomorphae (Order Mugiliformes)
Superorder Blenniimorphae (Orders Gobiesociformes and Blenniiformes)

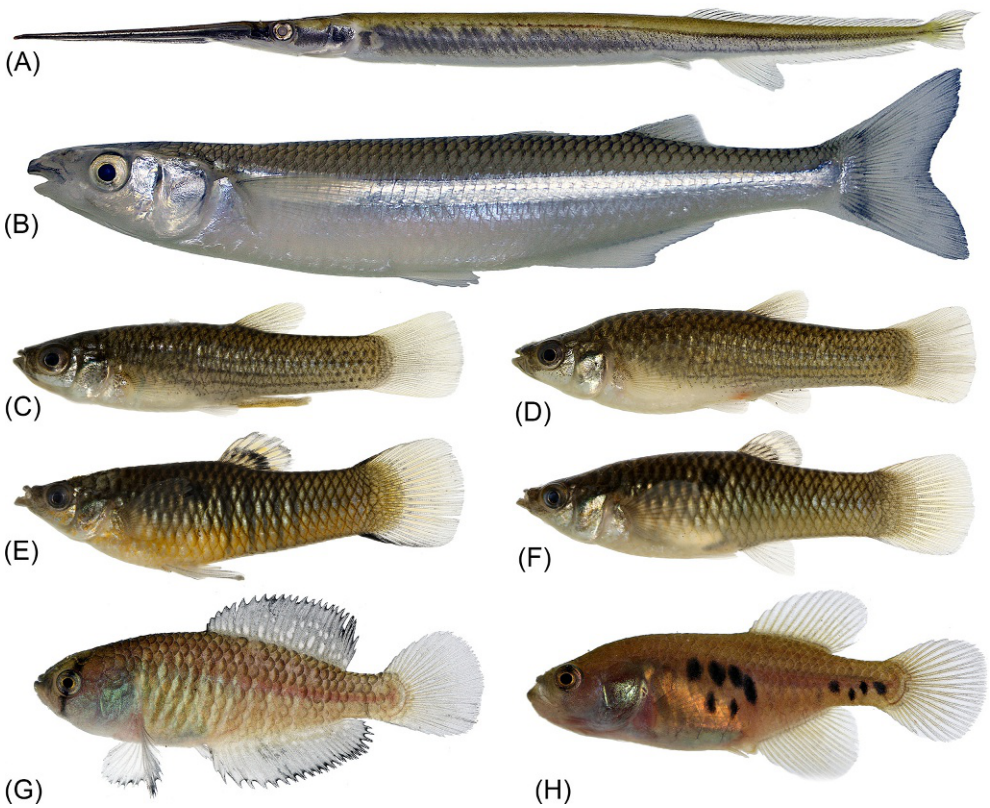

FIG. 1.12 Some representatives of Atherinomorphae: (A) Beloniformes, (B) Atheriniformes, (C–H) Cyprinodontiformes: Anablepidae male (C) and female (D), Poeciliidae male (E) and female (F), and Rivulidae male (G) and female (H). Not in scale.

The Cyprinodontiformes

The monophyly of the Atherinomorphae is based on a series of osteological, reproductive, and muscular characteristics, among others. Recent studies have shown that all the atherinomorphs, including Beloniformes (Fig. 1.12A), Atheriniformes (Fig. 1.12B), and Cyprinodontiformes (Fig. 1.12C–H), share a unique type of testis and ovary that, in turn, is correlated with a range of reproductive modifications, among which are spermatogenesis, internal fertilization, hermaphroditism, and viviparity (Parenti, 2005). They are mainly surface feeders and most of the more than 2000 species are confined to fresh or brackish waters.

The order Cyprinodontiformes (killifish) is the most diversified among the Atherinomorphae, with more than 1350 species distributed in temperate and tropical regions of the world. They usually inhabit shallow freshwater environments or coastal brackish waters, especially in Central America, making up about 13% of the Neotropical ichthyofauna. The small size of a maximum of a few centimeters, chromatic polymorphism, singular life cycle, complex behavior, and sexual dimorphism associated with an easy captive maintenance made them important models in experiments, and also very popular in aquariums

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(Costa, 1998). The Cyprinodontiformes are diagnosed by five synapomorphies, among which are the unlobed caudal fin, an epural symmetrically opposing to parhypural; and the first rib attached to the second vertebra rather than the third (Wiley and Johnson, 2010). The classification of the families in this order has changed in the last few years, actually including six families with representatives in the Neotropical region: Cyprinodontidae, Anablepidae (Fig. 1.12C and D), Poeciliidae (Fig. 1.12E and F), Rivulidae (alternatively referred as Cynolebiinae, a subfamily of Aplocheilidae in Costa, 2016, Fig. 1.12G and H), Fluviphylacidae, and Profundulidae.

Cyprinodontidae, at most 8 cm in length, have external fertilization and are distributed in freshwater, brackish, and coastal environments in the Americas, North Africa, and the Mediterranean region. Except for one species, the Anablepidae (four-eyes) have gonopodium formed by rays of the anal, associated with the sperm duct. Poeciliidae (livebearers barrigudos, guarus) have a laterally compressed body and no adipose fin, showing great variation in body size and shape. They are widely distributed in the Americas, and they are characterized by presenting anal rays modified in gonopodia, internal fertilization, and viviparity (Lucinda, 2003). Rivulidae can measure up to 20 cm, but most do not reach 8 cm and some species do not reach 3 cm. They have external fertilization and are oviparous, but two species have ovary and functional testes, being hermaphrodites that are self-inseminating. Many species are annual, that is, during drought periods, adults die and eggs remain in diapause in the substrate, only erupting in the next rainy season (Costa, 2003).

The Cichliformes

The Cichliformes order includes Cichlidae and Pholidichthyidae. The Cichlids form the richest family of nonostariophysean fish, including more than 1700 species and estimates that may reach 1900 (Kullander, 2003). Cichlids are distributed in Central and South America, the Antilles, Africa, Madagascar, South India, and Sri Lanka, inhabiting essentially freshwaters, but a few species occasionally enter brackish waters (Kullander, 1998). The more than 550 species of the Neotropical cichlids (Cichlinae) occur from the Rio Grande basin in North America to the Rio Negro basin in Patagonia, including the islands of Cuba and Hispaniola.

The cichlid ability to colonize and to diversify has been mainly explained by the advanced care with the offspring and the versatility of the pharyngeal and mandibular jaw apparatuses (Fig. 1.13). Besides the differentiation of the pharyngobranchial apparatus itself, the teeth vary in shape and size, reflecting different feeding specializations. Most cichlines also exhibit some type of sexual dimorphism in color pattern, body size, and in the development of structures such as humpbacks (Fig. 1.13E–H), crests, and fin-ray elongations. Due to this immense diversification, cichlids have long been the subject of massive ecomorphological, behavioral, biogeographical, and phylogenetic studies (Keenleyside, 1991; Winemiller et al., 1995). Phylogenetic analyses (e.g., Stiassny, 1991; Farias et al., 1999, 2000; Sparks and Smith, 2004) have recognized the family as monophyletic based on morphological and/or molecular evidence. Among the synapomorphies that support the monophyly of the family are: an extensive cartilaginous hood on the anterior margin of epibranchial 2; the head of epibranchial 4 expanded; microbranchiospines in the branchial gills; morphology of sagita; and short paired hypapophyses in the third and/or fourth vertebra (Kullander, 2003).

What novelties are expected in the phylogeny and classification of the Neotropical fish?

Knowledge on the diversity and evolution of Neotropical fish is continuously growing, concurring with the continuous development and exploration of new technologies and methods in the study of biodiversity. Due to the high biological diversity in the Neotropics and to the existence of undersampled areas or habitats in South and Central America, however, a higher number of discoveries are expected from this region, compared to other longer explored and studied regions of the world. Besides the description of new taxa, the increasing knowledge on the phylogeny of Neotropical fish is directly related to comprehension of the evolution of biological and ecological attributes of these organisms that ultimately allows understanding of the structure and functioning of freshwater ecosystems. Changes in classification should also be expected, perhaps more notably among the orders of the Euteleosteomorpha and subfamilies and genera of the Characidae. All these new discoveries make the study of the evolution of the Neotropical fish a productive and exciting issue for the next decades.

The updated number of species by family can be accessed through Eschmeyer's Catalog of Fishes at: http://researcharchive.calacademy.org/research/ichthyology/catalog/SpeciesByFamily.asp.

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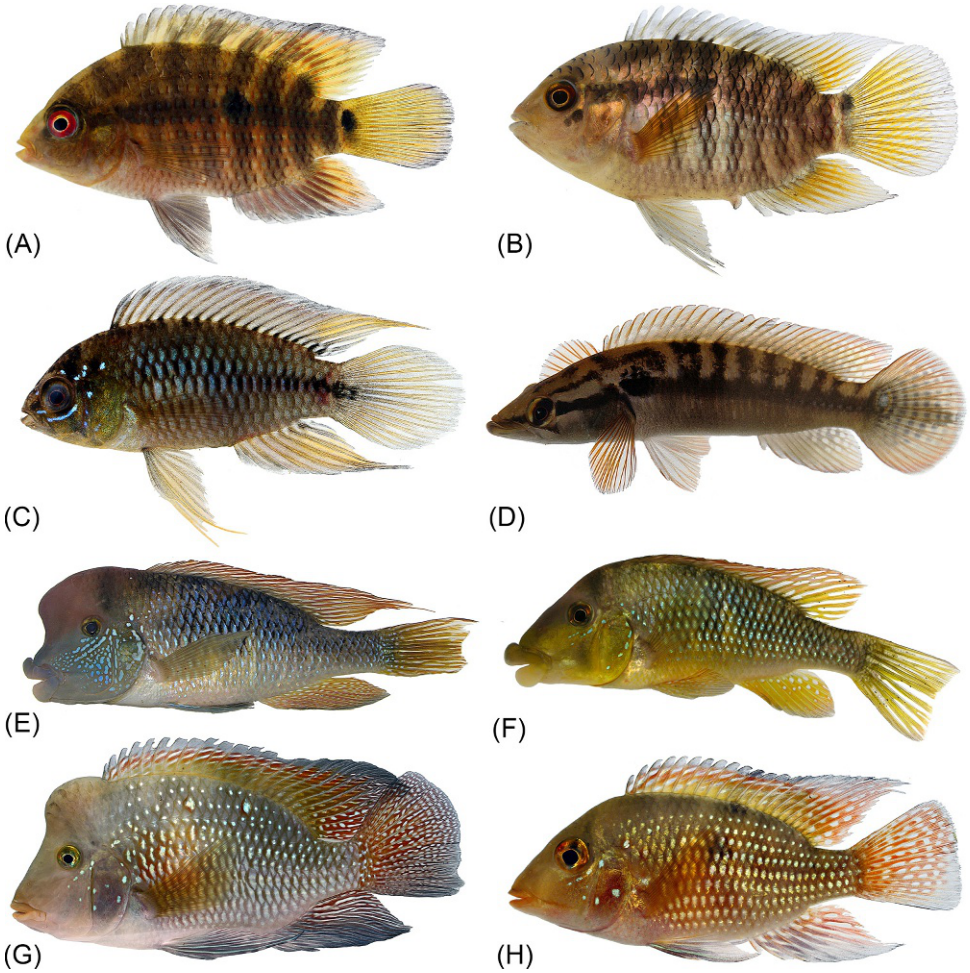

FIG. 1.13 Some representatives of the Family Cichlidae: (A) *Australoheros*, a Heroini, (B) *Cichlasoma*, a Cichlasomatini, (C–H) Geophagini: *Apistogramma* (C), *Crenicichla* (D), *Gymnogeophagus labiatus* male (E) and female (F), and *Geophagus brasiliensis* male (G) and female (H). Not in scale.

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Chapter 2

Anatomy of Teleosts and elasmobranchs

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Introduction

Fish is a noun, a term that defines aquatic, ectothermic, gill-breathing animals bearing bony—or cartilaginous-rayed fins (Gill and Mooi, 2004). Fish are a highly diverse group with more than 34,725 species comprising more than 50% of all vertebrates on the planet, of which about 1385 have cartilaginous skeletons (rays and sharks); 127 are Agnathans, that is, jawless fish (lampreys and hagfish); and the remainder are defined as Teleost fish, that is, fish bearing jaws and true bony skeletons (Eschmeyer and Fong, 2018). However, fish are not a monophyletic group, that is, unlike Tetrapoda—reptiles, birds, mammals, and amphibians—Teleost fish can be split into a primitive, an intermediate, and modern subgroups (Gosline, 1971).

The high diversity of the group evolved into many morphological types or body forms such as fusiform (conspicuous), compressed, depressed, truncated, attenuated, and anguilliform (see Lagler et al., 1977; Stoskopf, 1993) (Fig. 2.1). However, there is no "ideal" shape of form for a fish because each species has developed adaptations to fit its habitat and ecological niche. The understanding of aspects of fish anatomy becomes relevant from the moment in which its morphology (anatomy) is related to fish physiology, that is, the integration of the different systems.

This chapter will not describe the anatomical aspects of all Neotropical fish, which would be an impossible task given the group's biodiversity. This chapter dwells on the main anatomical features of Neotropical, freshwater Chondrichthyes (e.g., freshwater sting ray), Characins such as pacu (*Piaractus mesopotamicus*), Siluriforms such as silver catfish (*Rhamdia quelen*), and Cichlidae such as millet or pike cichlid (*Crenicichla* sp.).

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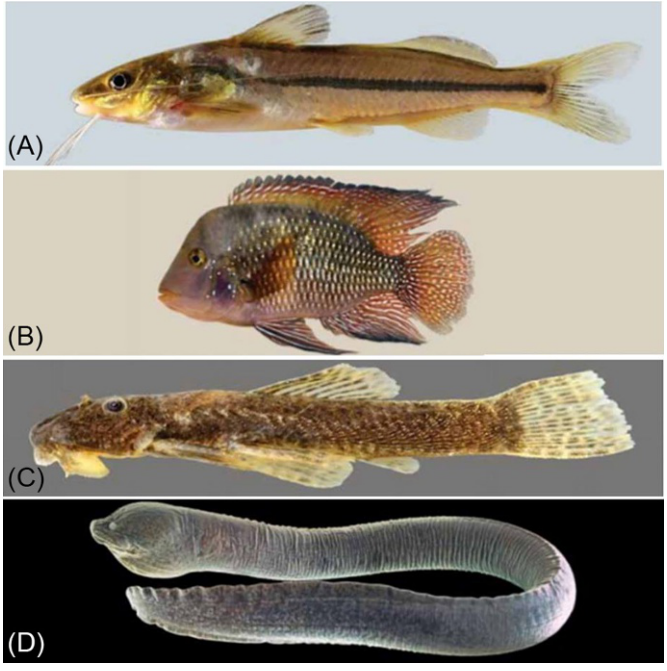

FIG. 2.1 Examples of species with different anatomical forms: (A) fusiform (*Pimelodella australis*), (B) laterally compressed (*Geophagus brasiliensis*), (C) dorsoventrally compressed (*Pareiorhaphis nodulus*), and (D) anguilliform (*Synbranchus marmoratus*). *(From Malabarba, L.R., Carvalho Neto, P., Bertaco, V.A., Carvalho, T.P., Santos, J.F., Artioli, L.G.S. 2013. Guia de identificação dos peixes da bacia do rio Tramandaí. Porto Alegre, Via Sapiens, with permission of the authors.)*

Common features of the external and internal anatomy of fish

Tegument

Similarly to vertebrates in general, the tegument—skin—of fish is a tissue covering the body of the animals. The skin not only separates and protects fish from the environment, but also enables animals to interact with the local environment and ecological conditions (Elliott, 2000). The skin of fish grows in importance when one considers the fact that in the aquatic environment, potentially pathogenic microorganisms have tridimensional distribution and occur in greater number per unit volume of water compared to the terrestrial environment per unit volume of the atmosphere (Dash et al., 2018).

The skin of Teleosts comprises a thin outer layer, the epidermis, and the dermis right below that. The epidermis is devoid of a keratinized layer, present in mammals, which enables constant development and multiplication of the cells (Morrison et al., 2006). The Teleost epidermis presents mucous cells all over the body surface. The mucus produced by these cells improves the body hydrodynamics, minimizing the water resistance during locomotion, allowing fish to swim at high speed with low energy cost (Elliott, 2000; Grizzle and Rogers, 1976).

The dermis of Chondrichthyes (elasmobranchs) evolved to produce high amounts of structural proteins, such as collagen, also prone to mineralization, which resulted in the development of a "dermal bone," and subsequently, the formation of scales and teeth (Meyer and Seegers, 2012). In addition, the dermis of cartilaginous fish is characterized by a layer system, a subepidermal extract called the *stratum laxum* where melanocytes are located. This characteristic accounts for the dark coloration of the epidermis of some freshwater stingrays, *Potamotrygon* sp. Below the dermis lies the hypodermis, lodging an extensive array of blood and lymphatic vessels, and adipose tissue, which is mainly connected to energy reserves during the breeding/spawning period (Whitear, 1986).

Elasmobranchs present a unique feature, the ampullae of Lorenzini (Fig. 2.2), a sensory system occurring in some regions of the body surface. Each ampulla consists of a cup-like structure connected to a channel filled with a gelatinous substance and a sensory epithelium composed of myelinated axons. The ampullae of Lorenzini enable elasmobranch fish to detect electric fields, thus allowing spatial orientation (navigation) and recognition of cohorts for reproduction as well as preys and predators (Wilkens and Hofmann, 2005).

Scales

The body of teleost fish is covered by somewhat tough skin armored by dermal structures, the scales, arranged in longitudinal and diagonal rows, except in scaleless fish such as the Siluriform fish (catfish). The type, number, and size of the scales provide relevant information on the biology of the species. The scales may vary from one layer of bony-ridged,

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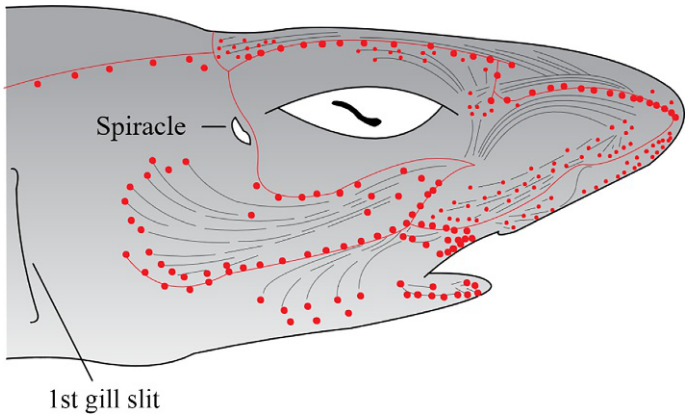

FIG. 2.2 Distribution of the sensorial system with the ampullae of Lorenzini in sharks. *(From http://commons.wikimedia.org/wiki/File: Electroreceptors_in_a_sharks_head-es.svg Accessed 11 April 2014.)*

flexible plates to a few plates covering only the caudal region of the fish (e.g., the mirror carp, *Cyprinus carpio specularis*) to thin and small scales covering the entire body or to the total absence of scales (Elliot, 2000). Modified scales in the form of bony plates or shields functioning as protective armor cover the body of certain species of fish such as the sturgeon (Acipenseridae) and many South American Siluriform (Loricariidae, Callichthyidae, and Doradidae); the number and size of the plates vary to a great extent between species (Fig. 2.3).

The front end of the scales of most bony fish is embedded in the skin, with a free posterior and exposed edges overlapping the next scale. Unlike placoid scales, these overlapping or imbricate scales are not replaced when lost, except in some cases of lesions (Laita and Aparício, 2005). The scales of bony fish are classified as cosmoids, ganoids, and elasmoids (cycloids and ctenoids).

- Cosmoid scales, described for crossopterigii and ancient dipnoi, are of dermal origin and deeply implanted in the dermis; cosmoid scales are the evolutionary precursors of the ganoid scales (Hildebrand and Goslow, 2001).
- Ganoid scales are characteristic of primitive, ray-finned fish, but in modern Teleosts they are restricted to a few species, such as the sturgeon (*Acipenser* spp.), the bichir (*Polypterus* spp.), and the gar (*Lepisosteus* spp.); ganoids are diamond-shaped and tightly compressed to each other.
- Elasmoid scales are the most common scales among bony fish, and are also restricted to this group (Bräger and Moritz, 2016; Hildebrand and Goslow, 2001); elasmoid scales are thin, translucent, and vary in shape (circular, oval, square), with two basic types:
- Cycloid scales, which have circular growth rings (*circuli*) and rays (*radii*) representing the sites of lower deposition of calcium salts during the formation of the scale; cycloid scales are conspicuous to the more "ancient" groups of bony fish (Fig. 2.4A).
- Ctenoid scales differ from cycloid scales because of the presence of minute spines (cteni) in their free or hind end. The function of the cteni is not yet fully understood, but there is evidence that they may improve the body's hydrodynamics, reducing trawling during swimming (Elliott, 2000). Ctenoid scales are usually present in more "recent" groups such as the Perciformes families Cichlidae, Soleidae, Percidae, Sparidae, etc. (Fig. 2.4B).

Elasmoid scales situated in the region of the lateral line have similar structures, but present a small pore communicating the mechanoreceptors of the lateral line channel to the environment. The number of perforated scales of the lateral line is a meristic feature frequently used in the classification and systematics of fish.

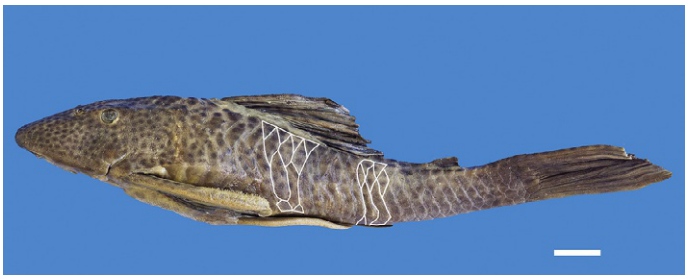

FIG. 2.3 "Cascudo," Siluriform of the family Loricariidae. Detail of the modified scales in the form of plates. Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

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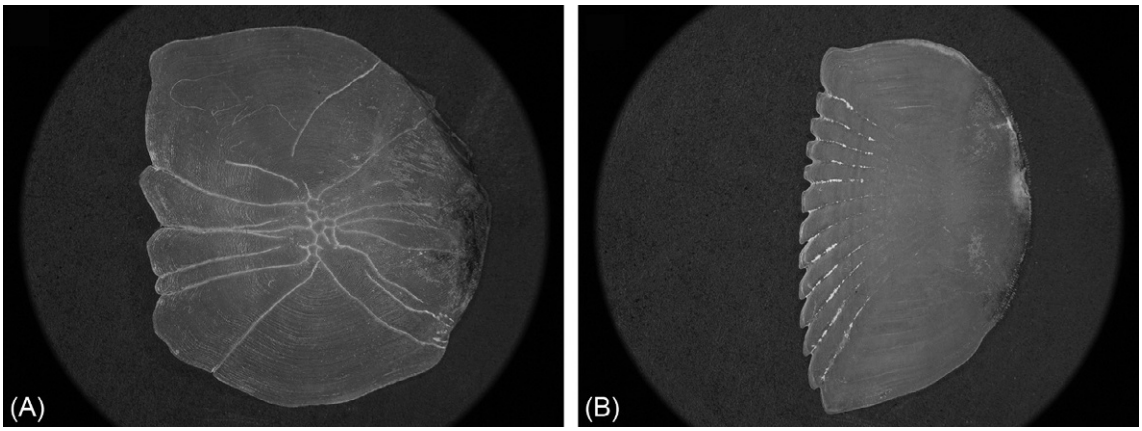

FIG. 2.4 Structure of cycloid (A) and ctenoid (B) scales. *(Photo: Everton Behr.)*

Because scales grow with fish growth, they can be used to determine the age of the fish through the observation and record of growth rings (Laita and Aparício, 2005), occurring when there is reduced calcium deposition as a result of reduced growth rate during food shortage or reproductive migration and spawning, for instance.

The elasmobranchs (sharks and rays) have placoid scales, which are rather small and end in a caudally oriented spine or denticule that gives the sensation of roughness to the touch (Laita and Aparício, 2005). In most sharks, these small scales cover the entire body surface, fins and gill slits included (Meyer and Seegers, 2012). Placoid scales are characterized by a blunt bony structure, acellular at the tip of each scale and aligned with the direction of the water flow. Placoid scales represent a modern version of the integument surface of an ancestor of sharks bearing bony armor. The morphology of placoid scales of elasmobranchs assists swimming, reducing water resistance and turbulence (Dean and Bhushan, 2010). In addition to improving hydrodynamics, the tooth-like structure of placoid scales provides protection against predators and ectoparasites (Southall and Sims, 2003). The autoecology of species also influences the morphology of the scales of the elasmobranchs. Bottom dwellers have larger scales and a depressed body while active pelagic species have longline-like scales, arranged as "tiles embedded in a roof" (Southall and Sims, 2003).

Fins

As a rule, fish present pectoral, pelvic, dorsal, anal, and caudal fins (Fig. 2.5). Several Characins and Salmoniform (trout and salmon) also have a small, ray-free adipose fin placed between the dorsal and the caudal fin (Fig. 2.5B and D), deemed

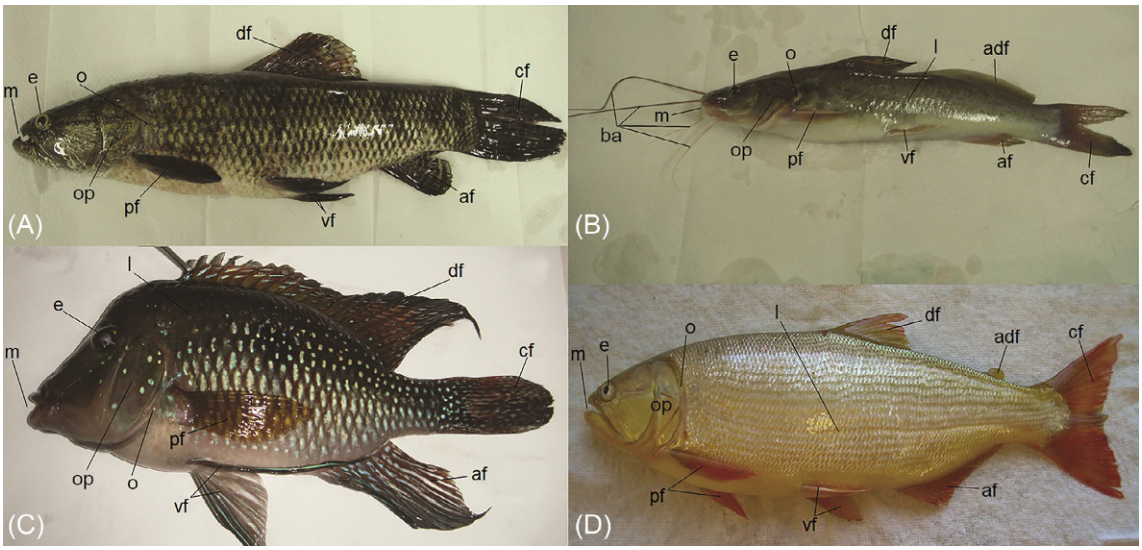

FIG. 2.5 Lateral view of (A) *Hoplias malabaricus*, (B) *Rhamdia quelen*, (C) *Geophagus brasiliensis*, (D) *Salminus brasiliensis*. *adf*, adipose fin; *af*, anal fin; *b*, barbels; *cf*., caudal fin; *df*, dorsal fin; *e*, eye; *l*, lateral line; *m*, mouth; *o*, opercular opening; *op*, operculum; *pf*, pectoral fin; *vf*, ventral fin. *((A and D) Courtesy by Fernando J. Sutili. (B and C) Courtesy by Ana Paula G. Almeida. Reprinted with permission of the authors.)*

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an ancestrality characteristic of these groups. Some fish, such as mackerel (*Scomberomorus cavalla*), have several small ventral and dorsal fins—finlets—between the dorsal and anal fins and the caudal fin (Moyle and Cech, 1988).

Fish use their paired pectoral and pelvic (or ventral) fins for balance and maneuvers in the aquatic environment (Yamanoue et al., 2010). Bottom dwellers such as the armored "viola" catfish (*Loricariichthys* spp.) present pectoral fins parallel to the body, enabling the fish to cling to substrates thus helping them to stand rapid water flow (Laita and Aparício, 2005). Pelvic fins may be absent in some species such as the moonfish (*Mola* sp.). The dorsal and anal fins work as keels, eliciting vertical stability—protecting fish against rolling—and maneuverability to fish, helping with sudden stops and changes in direction. The caudal fin, located at the end of the caudal peduncle, is the main propulsion appendage of fish, working along the body's undulation. Full body curling or undulation may be the sole displacement mechanism in fish such as the South American marbled eel (*Synbranchus* spp.).

Fins of fish stand on bony or cartilaginous rays—lepidotrichia. Some fins have soft rays while others have hard rays or spines. There are also species that bear spines, usually in the head end, and soft rays, usually in the caudal end of the fins. Soft rays are frequently branched (Yamanoue et al., 2010).

The anal fin rays of some male Characins such as *Astyanax* spp. and *Salminus* spp. do work as transient sex dimorphism features presenting small "hooks" during the reproductive season, bringing on roughness to the touch (Casciotta et al., 2003). The anal fin rays of males of species that present internal fertilization (e.g., Poecilidae (Lucinda, 2003) and Auchenipteridae (Ferraris Jr., 2003)) are modified into gonopodia, whose function is to inseminate the females (Laita and Aparício, 2005).

A dense skin covers the fins of cartilaginous fish so that the rays cannot be seen, unlike what occurs with bony fish. Furthermore, because elasmobranchs are devoid of gas bladders, their fins exert a greater effort toward vertical movement (Yamanoue et al., 2010). The internal parts of the pelvic fins of elasmobranchs are transformed into a copulatory organ, the clasper or pterygopod, present in male sharks and rays (Hildebrand and Goslow, 2001).

Rays are skate-shaped, so their pectoral fins are large and extend from the head to the pelvis along the trunk. Most of these fish have two median dorsal fins, which do not exist in the thorns rays, which also do not have anal fin, most of them not having a caudal fin either (Fig. 2.6). Freshwater rays (sting rays) hatch fully formed, the only difference being the presence of the yolk sac (Fig. 2.7). Some species have a thorn or sting in the tail that can cause serious injury to opportune predators, humans included, but in this case most by accident. Flanks of the anal fin of freshwater sting rays are very large, and work in conjunction with the dorsal fin for displacement purposes.

Fish caudal fins can be continuous or bilobed. Continuous (truncated) fins may have a rounded, straight, or slightly forked edge. According to their anatomy, fins can be classified into two types (Lagler et al., 1977). The first is protocercal, which is featured in cyclostomes and is rounded with a notochord extending straight to the posterior end (Fig. 2.8B). The second is diphycercal, featured in Dipnoi and lungfish such as the South American lungfish or "piramboia" (*Lepidosiren paradoxa*), which is fused to and continuous with the anal and dorsal fins (Fig. 2.8D).

Bilobate fins can be homocercal when the two lobes are of similar size (Fig. 2.8C), or heterocercal, which has a larger dorsal lobe within which extends the posterior part of the axial skeleton, and a smaller ventral lobe. Heterocercal fins are typical of sharks and are also present in sturgeons (Fig. 2.8A).

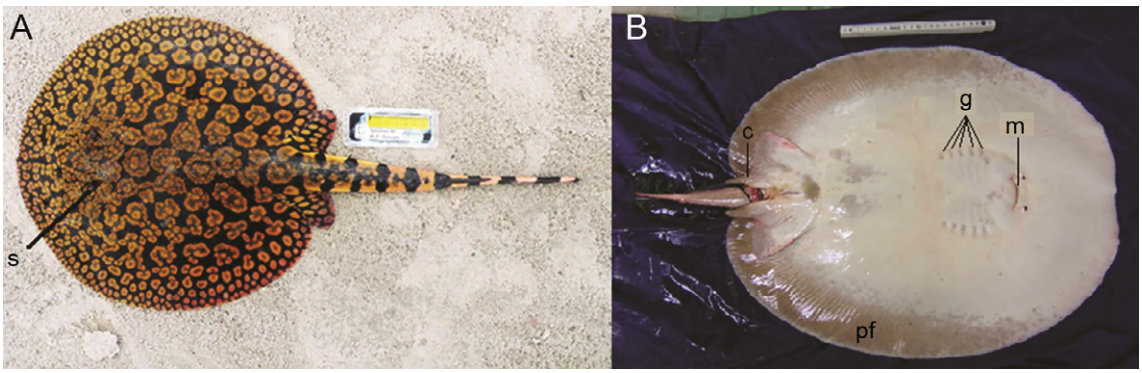

FIG. 2.6 (A) dorsal view of *Potamotrygon schroederi*. (B) ventral view of *Paratrygon aiereba* (male). *c*, clasper; *g*, gill slits; *m*, mouth; *pf*, pectoral fin; *s*, spiracular opening (spiracle). *(Courtesy of Wallice P. Duncan. Reprinted with permission of the author.)*

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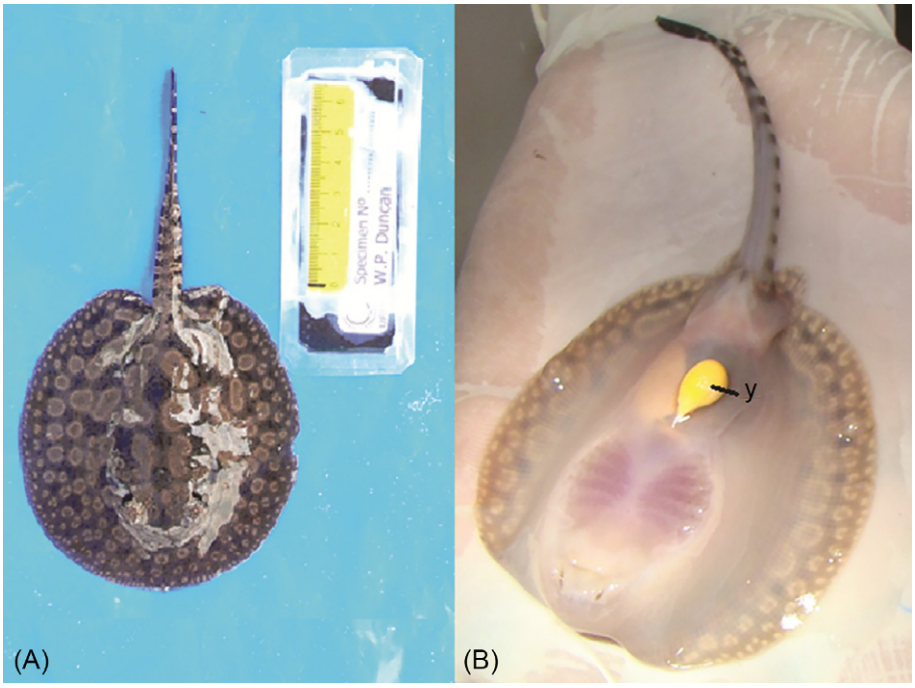

FIG. 2.7 *Potamotrygon* sp. (cururu ray), (A) dorsal view, (B) ventral view. *y*, yolk sac. *(Courtesy of Wallice P. Duncan. Reprinted with permission of the author.)*

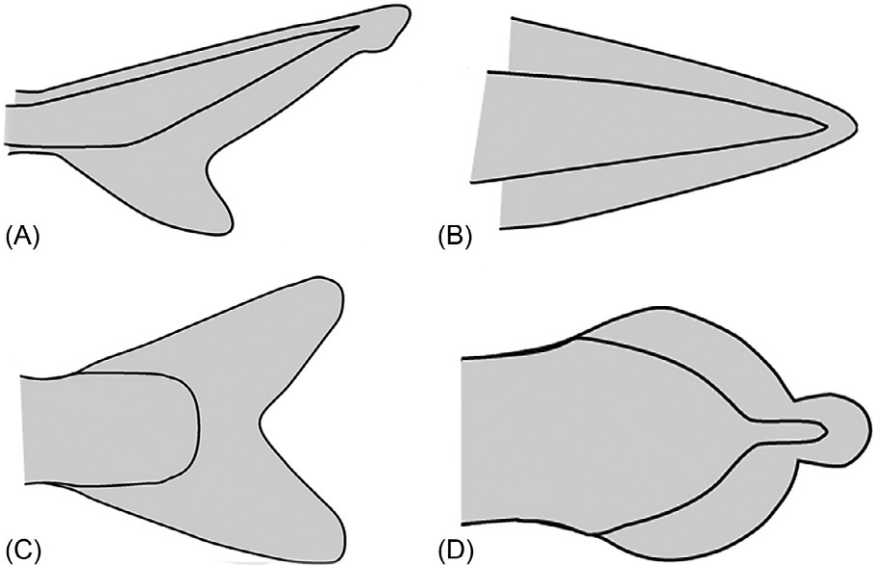

FIG. 2.8 Types of caudal fins of fish. (A) heterocercal, (B) protocercal, (C) homocercal, and (D) diphycercal. *(Reproduced from http://commons.wikimedia.org/wiki/File:PletwyRyb.svg Accessed 12 June 2018. Public domain.)*

General anatomy of Teleosts and elasmobranchs

Digestive system

Several organic systems of fish went through morphological and functional changes during the evolutionary process. This observed phenomenon particularly affected the digestive tract of fish, which underwent morphological modifications to allow species to explore a wide range of food niches and items (Buddington and Kuz'mina, 2000). As a rule, digestive systems of fish comprise the mouth and teeth, esophagus, stomach (absent in some species, e.g. common carp), intestine (with or without pyloric ceca), and accessory organs such as the liver, gallbladder, and pancreas (Figs. 2.9 and 2.10).

The position of the mouth is associated with the food habit and position in the water column. The mouth may be in the ventral position (bottom dwellers such as stingrays and armored catfish) (Fig. 2.11), terminal (nektonic fish, e.g., *Astyanax*

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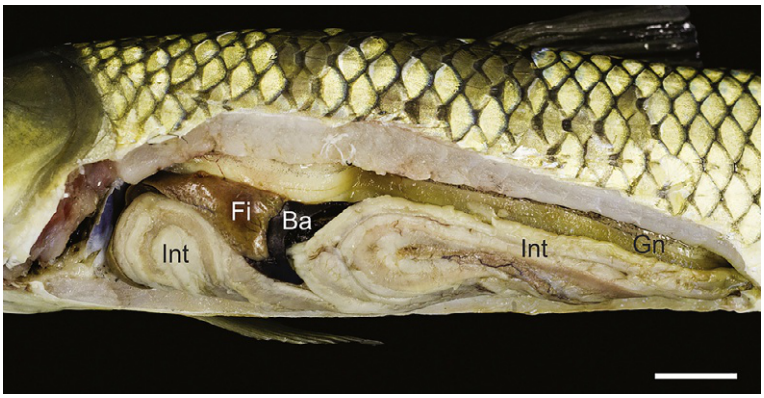

FIG. 2.9 Left lateral view of the digestive system of the grass carp, *Ctenopharyngodon idella*, a species that does not have stomach. Liver (Fi), intestine (Int), gonad (Gn), spleen (Ba). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

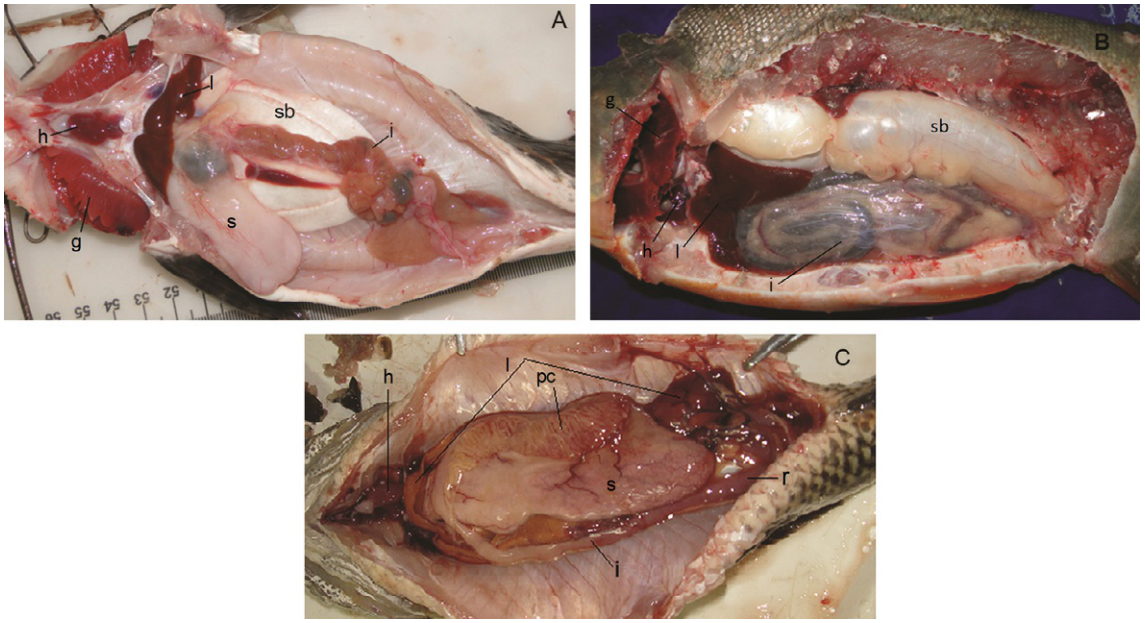

FIG. 2.10 (A) ventral view of the coelomic cavity of *Leiarius marmoratus*. (B) lateral view of the abdominal cavity of *Piaractus brachipomus*. Intestine still wrapped by the peritoneum. (C) Ventral view of the abdominal cavity of *Hoplias malabaricus*. *g*, gills; *i*, intestine; *l*, liver; *s*, stomach; *sb*, swim bladder. *(Courtesy of N. E. Cruz-Casallas (A and B) and Ana Paula G. Almeida (C). Reprinted with permission of the authors.)*

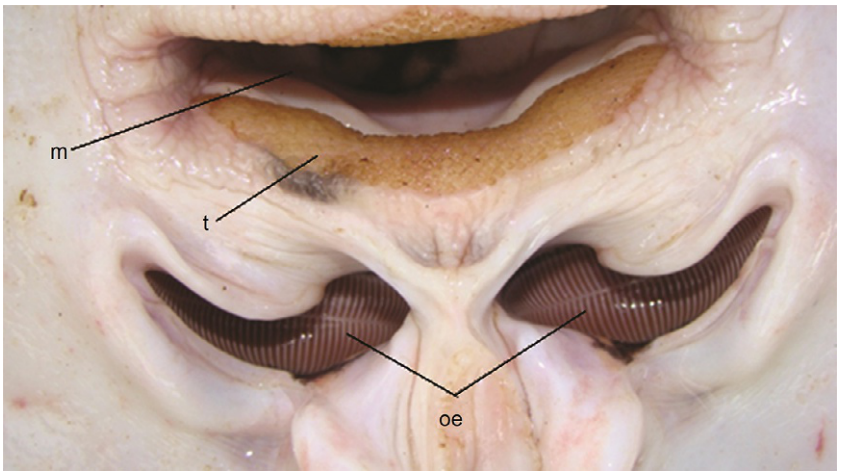

FIG. 2.11 Ventral view of *Potamotrygon motoro* showing the mouth (m), teeth (t), and nasal aperture or nostrils with the olfactory epithelium (oe) (part of the body surface was raised for visualization of the epithelium). The secondary folds of this epithelium form structures similar to the gill filaments, but the stratified epithelium is very thick. *(Courtesy of Wallice P. Duncan. Reprinted with permission of the author.)*

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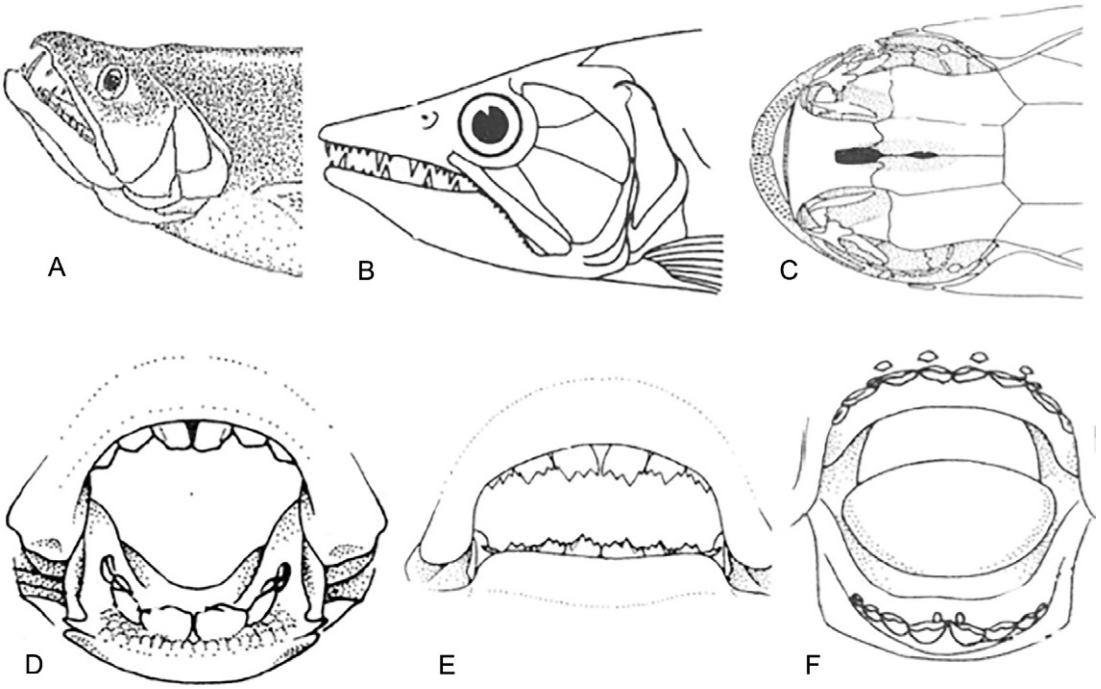

FIG. 2.12 Caniniform teeth in *Raphiodon vulpinus* (A) and *Acestrorhynchus* spp. (B), villiform teeth in Siluriformes (C), incisiform teeth in *Leporinus* spp. (D) and *Schizodon* spp. (E), and molariform teeth in *Metynnis* spp. *(Reproduced from Sampaio, A.L.A., Goulart, E. 2011. Ciclídeos Neotropicais: ecomorfologia trófica. Oecol. Aust. 15 (4), 775–798, with permission of the authors.)*

spp. and wolf fish, *Hoplias* sp.) or facing upward (surface fish, e.g., arowana, *Osteoglossum* spp., and "dourado-cachorro," *Rhaphiodon vulpinus*). Many Cichlidae and Cyprinidae fish have protractile jaws (Bone and Marshall, 1982) while other fish perform a kind of mouth sucking at the moment of imprisoning the prey (Pough et al., 2018).

Not all fish have teeth. For instance, teeth are absent in Curimatidae after the larval phase (Vari, 2003). Some species have canine teeth, specialized to hold prey such as the carnivorous *Hoplias* spp., *R. vulpinus*, and *Acestrorhynchus* spp. (Fig. 2.12A and B). Other fish, such as most Siluriform, have only one dentition plate formed by small, villiform teeth (Figs. 2.12C and 2.13). Species of the genera *Leporinus* spp. and *Schizodon* spp. have incisive-like teeth while *Metynnis* spp. have a small number of molariform teeth (Britski et al., 2007) (Fig. 2.12D–F). Some species present teeth on the palate (e.g., *Pygocentrus* spp.); some present teeth external to the mouth, as is the case of the lepidophagous species *Roeboides* spp.) (Britski et al., 2007); and some fish do have pharynx teeth, as is the case of grass carp, *Ctenopharyngodon idella*, and the Minkley's cichlid, *Herichthys minckleyi* (Sampaio and Goulart, 2011).

Gill arches are made on one side by the gill filaments, which carry out gas exchanging functions, and on the other side by the gill rakers, a fringe-like structure that filters suspended food items encompassed in the feeding habit of the species.

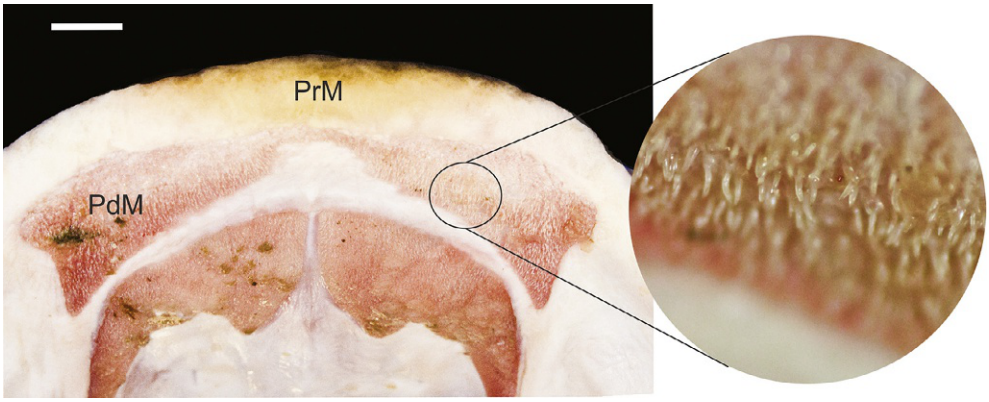

FIG. 2.13 Oral cavity of spotted sorubim *Pseudoplatystoma* sp. demonstrating maxillary dentigerous plaques. In the detail, small teeth of viliform shape. Premaxilla (PrM), maxillary dentition plaque (PdM). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

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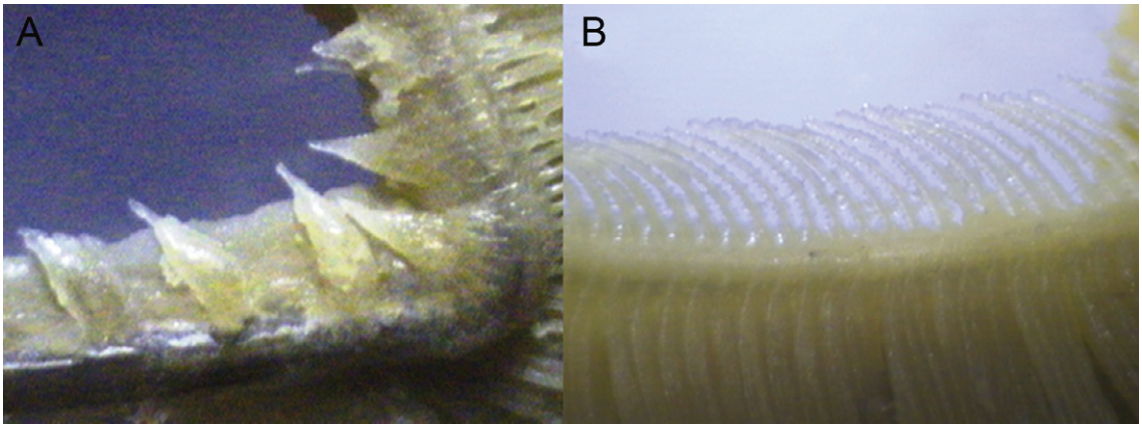

FIG. 2.14 (A) first branchial arch of *Serrasalmus maculatus*, with emphasis on the few and separated short rakers. (B) second branchial arch of *Parapimelodus nigribarbis*, with emphasis on long, numerous, and close rakers. *(Courtesy by Ana Paula G. Almeida. Reprinted with permission of the author.)*

Carnivorous fish have short, separated, cuspid teeth-like gill rakers whereas suspension, filter-feeding fish have numerous and long gill rakers (Fig. 2.14).

The digestive tracts of elasmobranchs and sturgeons have a spiral valve, a convolute structure that increases the absorption surface of the fish's digestive tract without the need to increase the intestinal length or to decrease the gastric transit time (Wilson and Castro, 2011). Further details on the digestive systems of fish are discussed in Chapter 11.

Respiratory system

Fish breathe through the gill filaments. These are red-colored, ridge-like folds covered by very thin lamellae anchored in cartilaginous arches housed in the gill chamber and covered by a flexible, mobile bony structure, the operculum, signaling the end of the fish head. Teleost fish have eight gill arcs, that is, four pairs within the buccal cavity (Olson, 2000). The anatomy of the respiratory system, the filament and gill lamellae organization, provides information about the biology of the species: animals with active swimming behavior have a larger gas exchange surface, which may also be related to fish that inhabit environments with low oxygen concentrations (Mazon et al., 1998).

Teleost fish present opercula whereas elasmobranch fish have gill slits instead. In the process of respiration, water enters through the mouth, passes through the gills, where oxygen diffuses from the water to the blood that circulates within the gill filaments, and then exits through the operculum or gill slits (Fig. 2.15). Further details on fish breathing mechanisms can be found in Chapter 10.

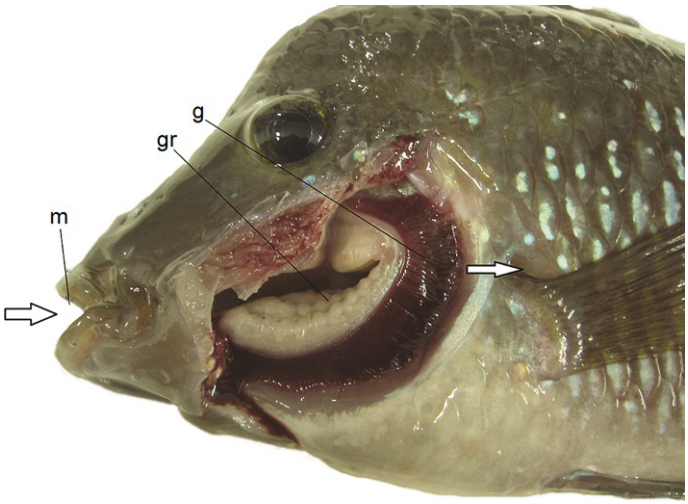

FIG. 2.15 Lateral view of *Geophagus brasiliensis*, with operculum removed for better visualization of gills (g) and gill rakers (gr). *Arrows* indicate the direction of the flow of water during respiration. *m*, mouth. *(Courtesy by Ana Paula G. Almeida. Reprinted with permission of the author.)*

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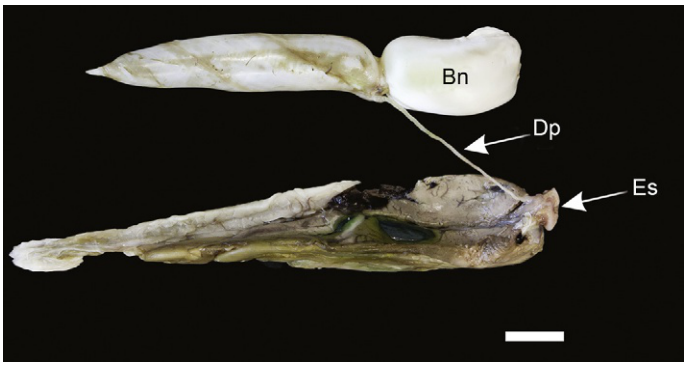

FIG. 2.16 Digestive system and swim bladder of grass carp, *Ctenopharyngodon idella*, a physostomous fish. Presence of the pneumatic duct connecting the swim bladder to the esophagus. Pneumatic duct (Dp), swim bladder (Bn), esophagus (Es). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

Swim (gas) bladder

The swim bladder present in most Teleosts lies right above the digestive tract and below the spinal vertebrae (consequently right below the kidney) and is beside the top portion of the pleural ribs. Swim bladders may be filled with either air or oxygen, thus playing a key role in maintaining neutral buoyancy and lowering energy costs for fish to remain at any certain depth (Helfman et al., 2009). In some species, such as the pirarucu, *Arapaima gigas*, the swim bladder is highly vascularized and functions as a respiratory organ (Brauner et al., 2004).

The swim bladder may be connected to the digestive tract, more specifically with the esophagus and stomach through a structure called the pneumatic duct (Fig. 2.16). According to this structure and the evolutionary pattern of the swim bladder, teleost fish can be grouped as physostomous (e.g., pacu, goldfish, carp) or physoclistous (e.g., Siluriformes in general). Physostomous fish maintain the connection of the swim bladder-esophagus all through the adult stage (Fig. 2.16), whereas physoclistous fish lose the pneumatic duct in the adult phase (Helfman et al., 2009).

Weberian apparatus

The Weberian apparatus, the hearing and balance system of fish, is a set of interconnected ossicles—tripus, intercalarium, scaphium, and claustrum—derived from the anterior vertebrae linked to a double chain of one to four ossicles that are the lower portion of the inner ear labyrinth, resting over the cephalic portion of the swim bladder. This system facilitates the transmission of sound, that is, the transmission of vibrations of the environment reverberated in the swim bladder into the inner ear, increasing the range of frequencies detected by fish and therefore improving the hearing perceptiveness (Fritzch, 2000; Helfman et al., 2009). The Weberian apparatus is found in Cypriniform, Characiform, Siluriform, and Gymnotiform fish (Diogo, 2009; Lechner and Ladich, 2008) (Fig. 2.17).

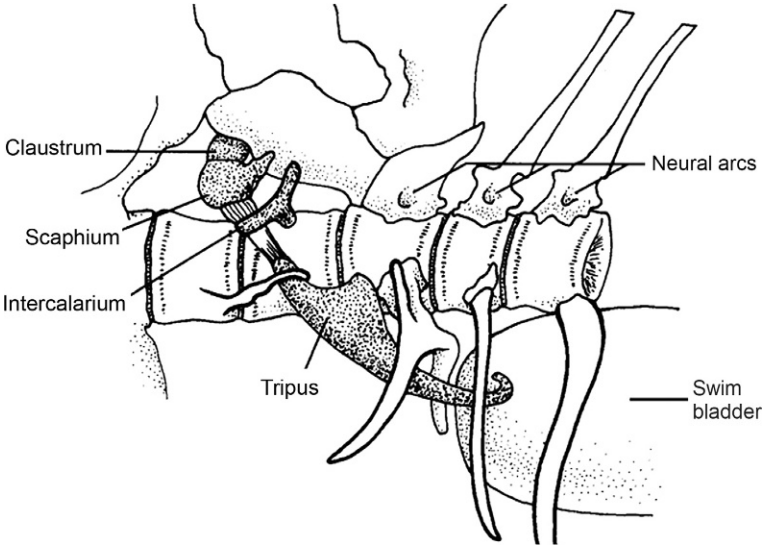

FIG. 2.17 Schematic illustration of the ossicles (Claustrum, Scaphium, Intercalarium and Tripus) that compose the Weber apparatus. *(Modified from Helfman, G.S., Collette, B.B., Facey, D.E., Bowen, B.W. 2009. The Diversity of Fish: Biology, Evolution and Ecology. Wiley-Blackwell, Oxford.)*

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Renal system

The renal system of Teleosts comprises the kidney, ureters, and urinary bladder. The kidney is located in the celomatic cavity (Fig. 2.18) right below the trunk vertebrae (i.e., between the transverse processes) and produces urine. Ureters (Fig. 2.18A) carry urine into the urinary bladder, where it is stored until excretion through the urogenital opening (Fig. 2.19). See Chapter 12 for further details on the functioning of the renal system.

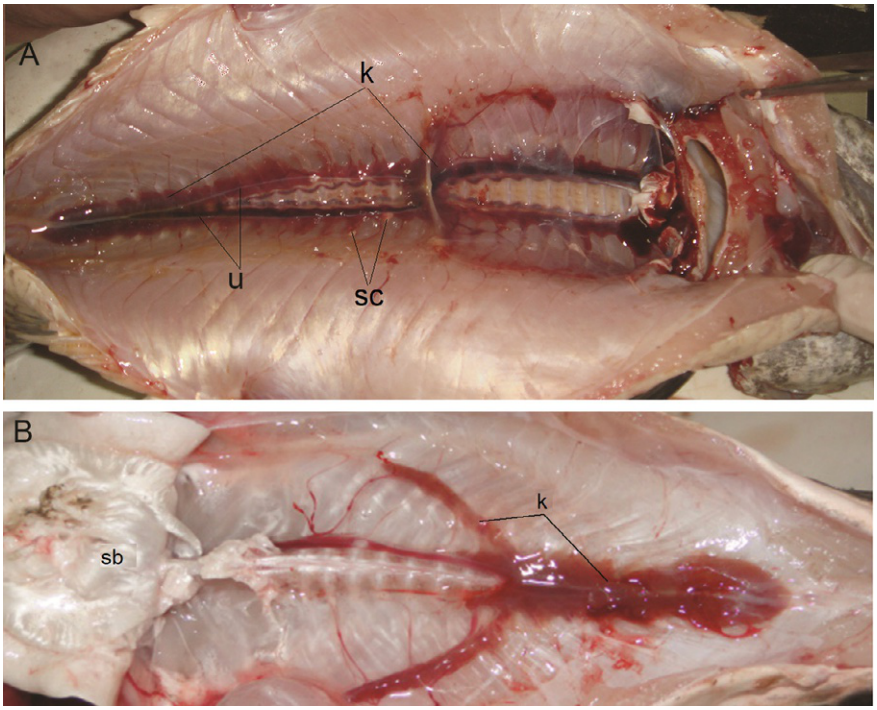

FIG. 2.18 Ventral view of (A) *Hoplias malabaricus* (B) *Leiarius marmoratus*. Digestive tract and swim bladder removed for visualization of the kidney. *k*, kidney; *sb*, swim bladder; *sc*, Stannius corpuscles; *u*, ureter. *(Courtesy by Ana Paula G. Almeida (A) and N.E. Cruz-Casallas (B). Reprinted with permission of the authors.)*

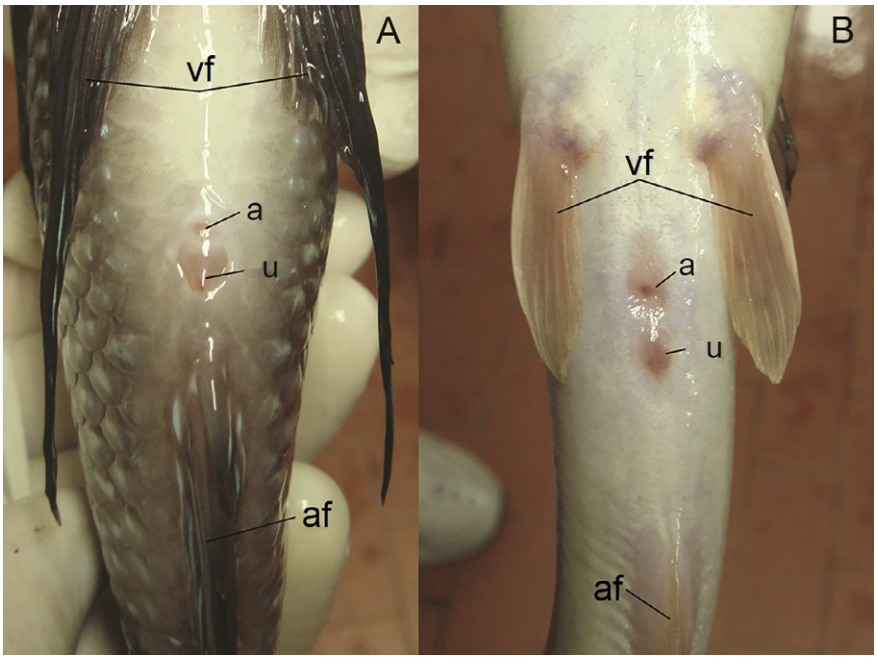

FIG. 2.19 Ventral view of (A) *Geophagus brasiliensis*, (B) *Rhamdia quelen*. *a*, anal opening; *af*, anal fin; *u*, urogenital opening; *vf*, ventral fin. *(Courtesy by Ana Paula G. Almeida. Reprinted with permission of the author.)*

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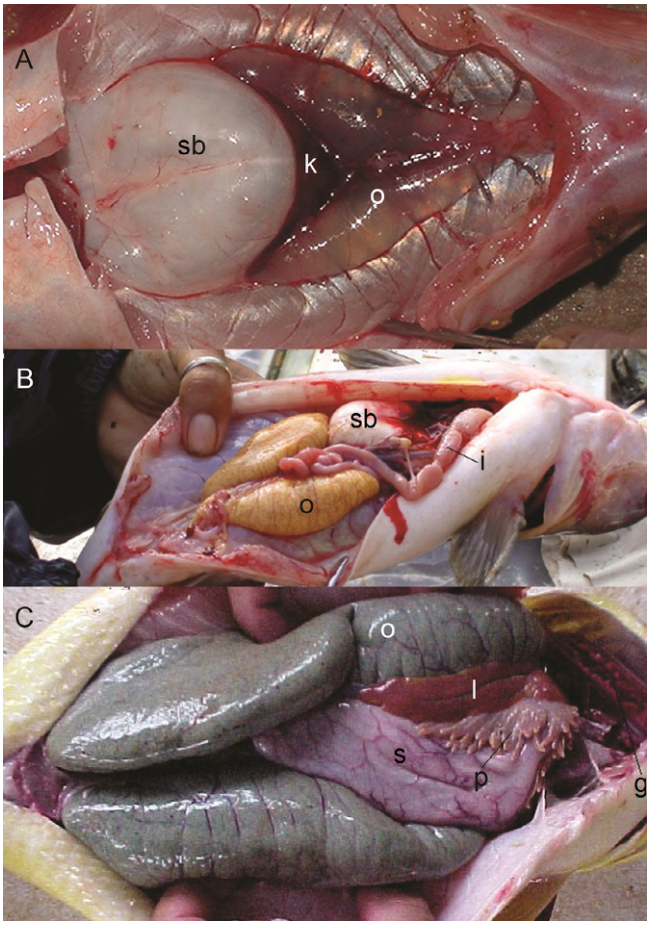

FIG. 2.20 Ventral view of females of *Rhamdia quelen* (A—maturing ovary, B—mature ovary) and *Salminus brasiliensis* (C—mature ovary). *k*, kidney; *l*, liver; *o*, ovarium; *p*, pyloric ceca; *s*, stomach; *sb*, swim bladder. *(Photos of Evoy Zaniboni Filho. Reproduced with permission of the author.)*

Reproductive system

Most Teleost are dioecious, that is, they have separate sex organs. Male Teleost bear testicles where the spermatozoa are produced while the females bear ovaries where the oocytes are produced (Redding and Patiño, 2000). Both ovaries (Fig. 2.20) and testicles (Fig. 2.21) appear in pairs. Detailed descriptions of the reproductive system of Teleosts are presented in Chapters 13 and 14.

The reproductive system of the elasmobranch male freshwater stingray entails a pair of testicles, an epigonal organ, a pair of epididymis (divided into the head, body, and tail), Leydig's gland, a pair of vas deferens, a pair of Marshall's alkaline glands, and a pair of claspers (Fig. 2.22). The reproductive system of a female stingray entails a pair of ovaries attached to the posterior oviducts or uterus by the ostia and anterior oviducts. The uterus ends in the urogenital sinus. Freshwater rays present placental viviparity with trophonemata, that is, a highly vascularized internal wall and villi (Fig. 2.23), with embryos developing properly in the uterus (Silva et al., 2017).

Nervous system

The nervous system of fish drives the integration with the (external) environment and the control of organs and systems. The perception or understanding of the surrounding environment allows the perfect coordination during displacement (i.e., swimming and migration), feeding, reproduction, and ordinary behavior. The integration between systems allows controlling all bodily functions and processes necessary for the survival and steadiness of the species. Although the nervous and the endocrine systems may be considered as working independently, they often work synergistically in the regulation of the organic responses to changes in the external and internal environments.

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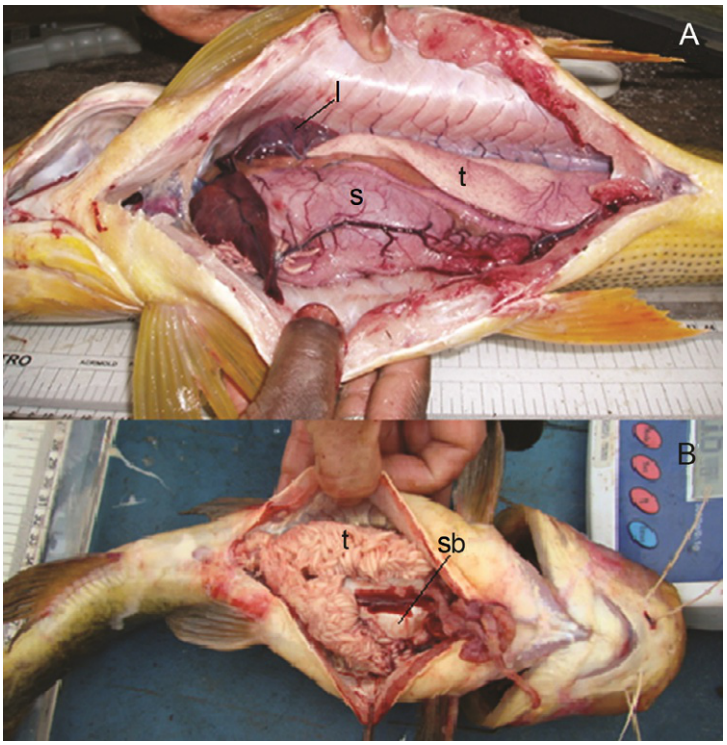

FIG. 2.21 Ventral view of males of *Salminus brasiliensis* (A) and *Rhamdia quelen* (B). *l*, liver, *s*, stomach; *sb*, swim bladder, *t*, testicles. *(Photos of Evoy Zaniboni Filho. Reproduced with permission of the author.)*

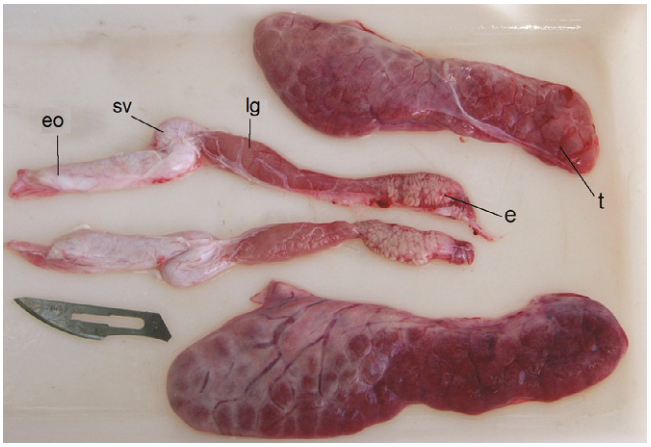

FIG. 2.22 Male reproductive apparatus of *Potamotrygon motoro*. *e*, epididymis; *eo*, epigonal organ; *lg*, Leydig's gland; *sv*, seminal vesicle; *t*, testicle *(Courtesy of Maria Lúcia Góes de Araújo. Reprinted with permission of the author.)*

The nervous system of fish can be parted into the central and peripheral nervous system. The central nervous system comprises the brain and spinal cord while the peripheral nervous system is made of nerves and ganglia. The functions of the structures of the central nervous system are defined in Table 2.1 (see also Fig. 2.24) (Bernstein, 1970; Genten et al., 2009; Butler, 2011; McLean and Dougherty, 2015; D'Elia and Dasen, 2018). The brain cavity is not clearly separated by the meninges to isolate the cerebrospinal fluid, but by connective structures with plenty of adipose tissue between the blood vessels and rich membranes covering the brain. The olfactory bulbs are positioned rostrally to the telencephalic hemispheres; some Characins such as the white cachama *Piaractus brachypomus* (Fig. 2.24) and the cardinal tetra *P. axelrodi* (Obando et al., 2013) do not present an olfactory tract. This characteristic could be related to a higher velocity of olfactory stimuli to reach the olfactory bulbs in Characins in comparison to Siluriform, which exhibit large olfactory tracts (Londoño and Hurtado, 2010). Nocturnal Siluriformes such as *Pseudopimelodus* spp. and *Spectracanthicus javae* have a large cerebellum and a relatively small *tectum opticum* compared to Characins (Chamon et al., 2018).

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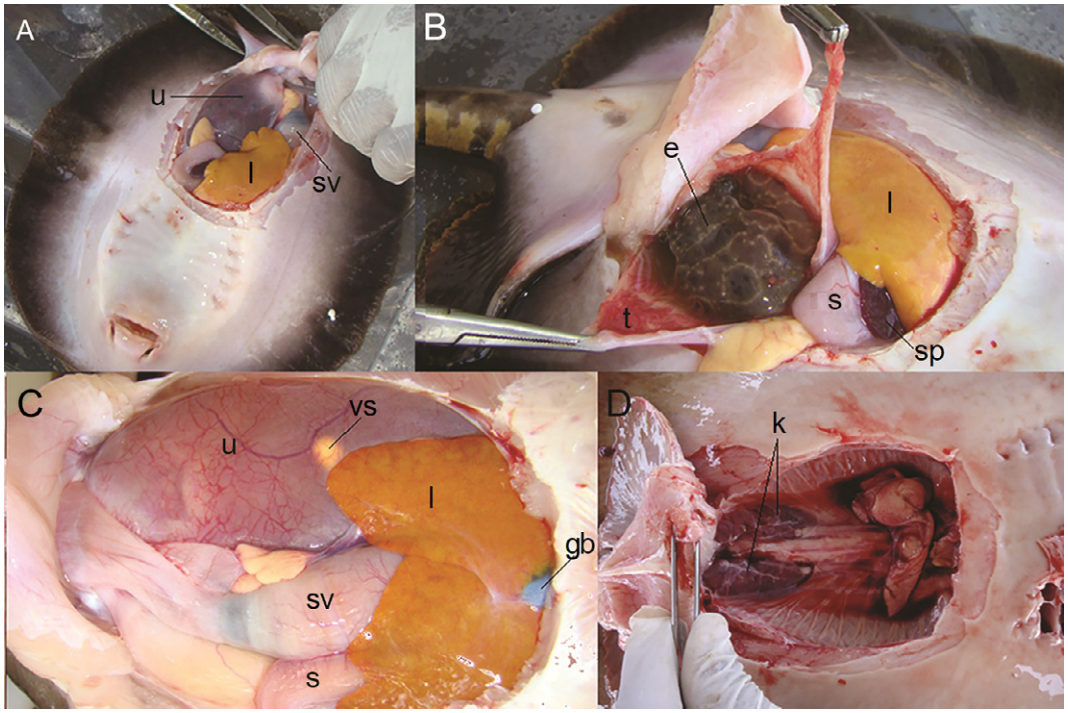

FIG. 2.23 Ventral view (A) of pregnant female of *Potamotrygon* sp. (cururu ray). In (B), trophonemata (t, uterine wall) removed for visualization of the embryo. In (C), internal organs and (D), several organs were removed for visualization of the kidneys (k). *e*, embryo; *gb*, gallbladder; *l*, liver; *s*, stomach; *sp*., spleen; *sv*, spiral valve; *u*, uterus; *vs*, embryo vitelline sac. *(Courtesy of Wallice P. Duncan. Reprinted with permission of the author.)*

The autonomic nervous system (ANS) is hold part of the peripheral nervous system. The classic division of the ANS in tetrapods comprises the sympathetic nervous system, the parasympathetic nervous system (cranial and sacral nerves), and the enteric nervous system (autonomic nerves intrinsic to the intestine). However, this division is not coherent for fish, so the terminology proposed by Nilsson (2011a) is herein adopted:

- Cranial autonomic system: parasympathetic pathways that follow the cranial nerves.
- Autonomic spinal system: sympathetic pathways parallel to the spinal cord and parasympathetic sacral pathways. In the elasmobranchs, the paravertebral ganglia are segmentally distributed parallel to the length of the spinal cord but not completely connected longitudinally, as in Teleosts.
- Enteric system: autonomic nerves intrinsic to the intestine (that is, maintain the definition used for mammals).

Fish have 10 pairs of cranial nerves (Nilsson, 2011a,b; Taylor et al., 2010):

I Olfactory: innervates the olfactory bulb, responsible for the transmission of olfactory impulses.
II Optical: innervates retina, transmits impulses related to vision.
III Oculomotor: innervates most of the muscles of the eye.
IV Trochlear: innervates the upper oblique eye muscle.
V Trigeminal: innervates the anterior portion of the head and the mandible and maxilla, transmitting motor and sensorial signals (thermal, tactile, and proprioceptive).
VI Abducens: innervates the posterior rectus muscle.
VII Facial and VIII auditory: can be considered as a facial auditory set, transmitting motor signals for some muscles of the head and sensorial (visceral, lateral, auditory, gravity, tactile, gustatory, proprioceptive).
IX Glossopharyngeal and X vagus: sometimes fused, lead sensory signals (lateral line, gustatory), innervate muscles related to breathing. The vagus nerve also transmits motor signals to the viscera.

Because of the absence of salivary and lacrimal glands in fish, the autonomic cranial pathways usually occur only in the cranial nerves III and X. The facial and glossopharyngeal nerves of elasmobranchs may also be autonomic; the autonomic cranial pathways of the South American lungfish *L. paradoxa* are restricted to the vagus nerve. Finally, a very particular feature is that the fish ANS also innervates chromaffin cells, which are inserted into the kidney.

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TABLE 2.1 Divisions of the central nervous system

Divisions		Functions
Spinal cord		Control of locomotion
Hindbrain or rhombencephalon	Medulla oblongata	It determines the basic rhythm and regulation of the respiratory and cardiovascular systems. It contains most of the motor and sensory cranial nerve nuclei. It is a place of passage of the neural pathways, making the connection between the spinal cord and the encephalon
	Cerebellum	It is related to precise and fast motion control, and in electric fish is related to the interpretation of electroreceptors
Midbrain or mesencephalon		Optic tectum: center for integration of visual information with other sensorial information
		Toris semicircularis: receives auditory (and sometimes electrosensory) input
		Tegmentum: participates in motor control
Forebrain	Diencephalon	Pretectum: receives retinal projections and is involved in the control of eye movements
		Epithalamus and pineal: pineal controls circadian rhythms and secretes melatonin
		Thalamus: promotes the filtration of sensory information
		Hypothalamus: control of thermoregulation, participates in the osmoregulatory control, food intake, emotional state, endocrine system
	Telencephalon	Pallium: receives and integrates sensory information
		Subpallium: motor control and related functions
		Amygdala: emotions
		Hippocampus: memory formation
		Olfactory bulb: interpretation of olfactory signals

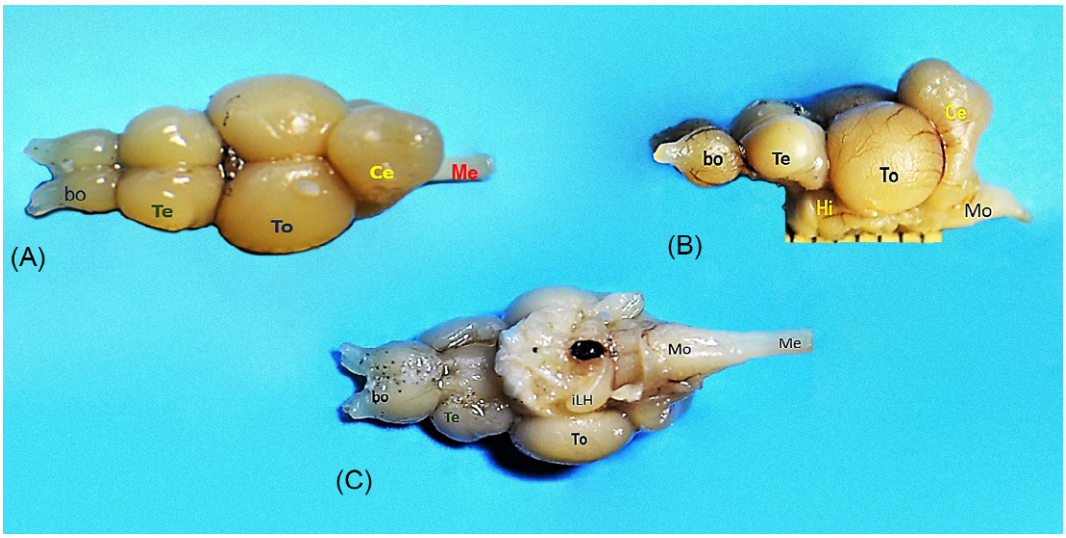

FIG. 2.24 Dorsal (A), lateral (B), and ventral (C) views of the central nervous system of juvenile *P. brachypomus* (40 g). *bo*, bulbus olfactorius (olfactory bulb); *Ce*, cerebellum; *Hi*, hypothalamus; *iHL*, inferior hypothalamic lobe; *Mo*, medulla oblongata; *S*, spinal cord; *Te*, telencencephalon; *To*, Tectum opticum. *Arrows* in (A), (B)—beginning of olfactory nerve, (C)—choroidal vascular structure, *sacum vasculosum. (Photo of Pedro René Eslava.)*

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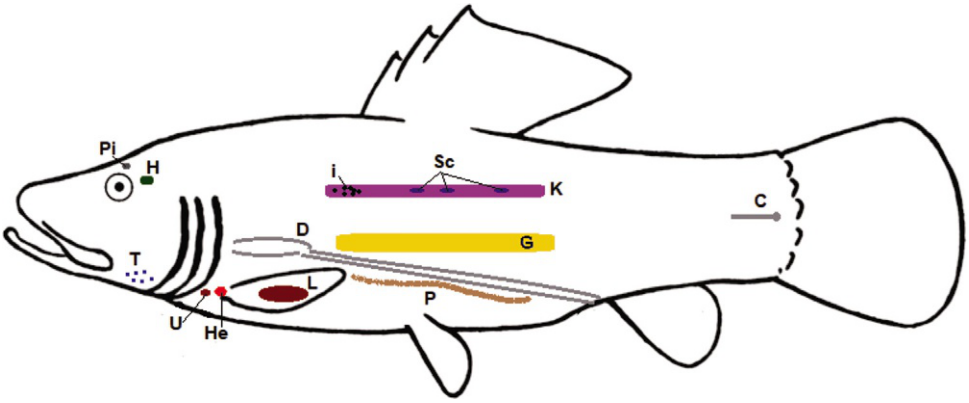

FIG. 2.25 Simplified representation of the localization of some endocrine structures. *C*, caudal neurosecretory system (urophysis); *D*, digestive tract; *K*, kidney; *G*, gonads (ovary or testis); *H*, hypothalamus-pituitary; *He*, heart; i, interrenal cells; *L*, liver; *P*, pancreas; *Pi*, pineal; *Sc*, Stannius corpuscles; *T*, thyroid follicles; *U*, ultimobranchial gland. *(Modified from Baldisserotto, B. 2013. Fisiologia de Peixes Aplicada à Piscicultura. EDUFSM, Santa Maria.)*

Endocrine system

The hypothalamus, which functions as an interface between the nervous and endocrine systems, resides ventrally to the thalamus in the lower portion of the brain (Fig. 2.25). The hypothalamus produces several hormones (Ogawa and Parhar, 2013; Biran et al., 2015), and is connected to another endocrine structure, the pituitary gland. The pituitary is parted into neurohypophysis and adenohypophysis, the last one comprising the rostral pars distalis, proximal pars distalis, and pars intermedia. Some neurosecretory cells of the hypothalamus have their axons ending at the neurohypophysis, which stores and releases the hormones produced by these cells (Table 2.2) to the rest of the body. Teleosts do not have the hypothalamic-pituitary-portal vascular system, which carries the blood passing through the hypothalamus directly to the adeno-hypophysis. However, hypothalamic neurosecretory cells release hormones that influence the production and release of adenohypophysial hormones (Table 2.2).

The adenohypophysis produces several hormones that act directly in some organs, but others regulate the production and release of hormones from endocrine glands (Whittington and Wilson, 2013; Martos-Sitcha et al., 2014. Prado-Lima and Val, 2015; Ah and Khairnar, 2018). These endocrine glands and other organs that release hormones are in Table 2.2 and Fig. 2.25.

TABLE 2.2 Main endocrine organs of Teleosts, hormones and their main functions

Organ	Hormones	Functions
Brain/ hypothalamus	Kisspeptin	Regulator of reproduction
Hypothalamus	Arginine vasotocin (AVT)	It stimulates spawning reflexes and reproductive behavior, adrenocorticotropic hormone (ACTH) release, contraction of the smooth muscle of the blood vessels of the gills, decreases the formation and elimination of urine
	Isotocin	It increases spermatozoids in sperm
	Melanin-concentrating hormone (MCH)	It stimulates the aggregation of pigments in melanophores, xanthophores, and erythrophores
	Gonadotropin-releasing hormone (GNRH)	It stimulates the release of gonadotropins
	Dopamine	It inhibits the release of gonadotropins
	Corticotropin-releasing hormone (CRH)	It stimulates the release of ACTH and melanocyte-stimulating hormone (α-MSH)
	Thyrotropin-releasing hormone (TRH)	It stimulates the release of thyrotropin and α-MSH
	Somatostatin	It inhibits the release of the growth hormone (GH)
	Growth hormone-releasing hormone (GHRH)	It stimulates the release of GH
	Prolactin-releasing peptide (or factor) (PRRP)	It stimulates the release of prolactin

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TABLE 2.2 Main endocrine organs of Teleosts, hormones and their main functions—cont'd

Organ	Hormones	Functions
Adenohypophysis	Growth hormone (GH)	It stimulates the secretion of insulin growth factors (IGF-I and IGF-II)
	Prolactin	It stimulates osmoregulatory adaptations to freshwater
	Somatolactin	It is related to physiological responses to stress, regulation of calcium, phosphate, and acid-base equilibrium
	Adrenocorticotropic hormone or corticotropin (acth)	It stimulates cortisol secretion and proliferation of interrenal cells
	Follicle-stimulating hormone or gonadotropin I (GTH I)	It stimulates estradiol release by the ovarium, gonadal growth, gametogenesis, and the vitellogenin uptake by the oocyte
	Luteinizing hormone or gonadotropin II (GTH II)	It stimulates final gamete maturation and release
	Thyroid-stimulating hormone or thyrotropin (TSH)	It stimulates thyroid growth and secretion
	Melanocyte-stimulating hormone (α-MSH)	It stimulates melanin production and pigment dispersion in the skin
	Parathyroid hormone-related protein (PTHRP)	Hipercalcemic effect
Thyroid	Triiodothyronine (T3) and thyroxine (T4)	They stimulate metabolism, growth, and metamorphosis
Chromaffin cells	Adrenaline (epinephrine) and noradrenaline (norepinephrine)	They stimulate physiological changes related to acute stress
Interrenal cells	Cortisol	immunosuppression, hyperglycemia, osmoregulatory adaptations to seawater
Pancreas	Insulin	It stimulates the synthesis and storage of nutrients in the cells
	Glucagon	Hyperglycemia and hyperlipemia
	Somatostatin	It reduces gastrointestinal secretions
	Amilin	Anorexigenic action
Gastrointestinal tract	Bombesin, gastrin, ghrelin, cholecystokinin, secretin	Movements and secretions of gastrointestinal tract
Heart	Natriuretic peptides (or factors	Vasodilation and reduction of blood pressure, also stimulates diuresis
Pineal	Melatonin	Synchronization of reproductive period
Ultimobranchial gland	Calcitonin	Hypocalcemia
Stannius corpuscles	Stanniocalcin	Antihypercalcemic
Caudal neurosecretory system	Urotensin I (UI)	It stimulates ACTH and cortisol release, freshwater adaptation (?)
	Urotensin II (UII)	It stimulates contraction of smooth muscles, freshwater adaptation (?)

General anatomy of Neotropical Characins, Siluriform, and Cichlidae

Pacu *Piaractus mesopotamicus* (Holmberg, 1887)

The "pacu," *P. mesopotamicus* (Ostariophysi: Characiforme: Serrasalmidae), is a South American Characin native to the Paraná, Paraguay, and Uruguay River basins (Pelicice et al., 2017; Scarabotti et al., 2017). The species has a tall and compressed body covered by small scales, pelvic fins in the abdominal position, and an adipose fin (Fig. 2.26).

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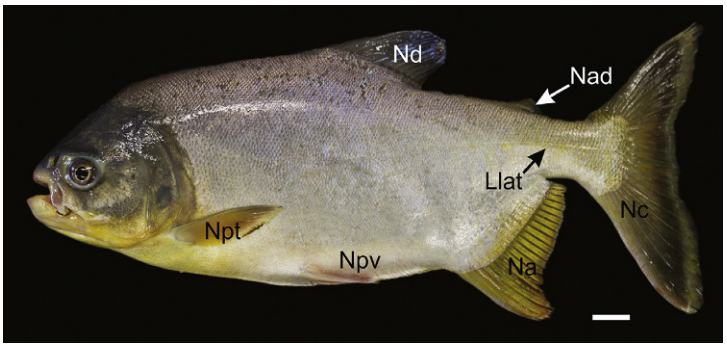

FIG. 2.26 External morphology of pacu (*Piaractus mesopotamicus*). Dorsal fin (Nd), pectoral fin (Npt), pelvic fin (Npv), anal fin (Na), caudal fin (Nc), adipose fin (Nad), lateral line (Llat). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

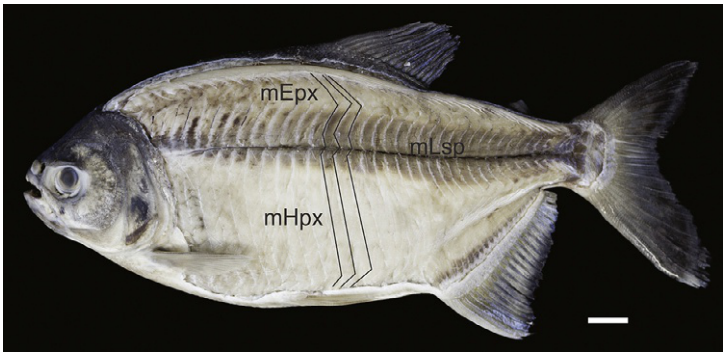

FIG. 2.27 Left side view of the muscle tissue of pacu (*Piaractus mesopotamicus*) and delimitation of its different anatomical regions. Details of myomeres arranged in a "W-shaped" disposition. Epaxial myomere (mEpx), hypaxial myomere (mHpx), *Lateralis superficialis* muscle (mLsp). *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

The muscular system of "pacu" is characterized by W-shaped, piscine myomeres. As a rule, fish muscles are grouped according to their location and biochemical characteristics (Fig. 2.27). The epaxial and hypaxial myomeres practically form the entire musculature of a fish's body. Epaxial myomeres located at the fish's caudal region play an important role in propulsion and swimming, whereas those located at the cranial regions are related to the feeding processes because their contraction causes the expansion of the orobranchial chamber (Stiassny, 2000). The *Lateralis superficialis* muscle is layered at the median region of the body, covering horizontally all the fish's length. It comprises a tissue rich in myoglobin, which provides a darker coloration compared to the rest of the musculature with a higher number of mitochondria, blood vessels, and lipids. Therefore, this muscle is associated with a high metabolism and maintenance of swimming at high speeds for long periods; it is found in higher quantities in fish with active swimming behavior (Stiassny, 2000).

The muscle fibers responsible for moving dorsal and anal fins derive from the epaxial (dorsal) and hypaxial (ventral) muscles and can be parted into dorsal and anal erector, depressor, and inclining muscle fibers. Their activity is related to the lateral stability maintenance (Chadwell and Ashley-Ross, 2012).

Pacu is an omnivorous, broad-spectrum euryphagous or opportunistic species that feeds mostly on leaves, stems, flowers, fruits, and seeds from plants of the riparian region of water bodies. However, it can opportunistically feed on a variety of small invertebrates in general and other small fish (Urbinati et al., 2010). Pacu has molariform dentition (Fig. 2.28), specialized in the crushing and grinding of hard food items such as fruits and seeds (Britski et al., 2007).

The morphology, placement and function of the gills of pacu—lamellae and grill rakers—follow the general patter of Characin species, situated in the orobranchial chamber and protected by the operculum (Fig. 2.29). The topographical location of the digestive system—stomach, pyloric ceca, liver, gallbladder, and the cranial portion of the left and right intestinal loops, extending all the way to the anus (cranial and caudal intestine or intestine I and II; Fig. 2.30)—can be detected in the visceral cavity. The gonads are situated dorsally to the final position of the intestine and rectum; the swim bladder lies a little above them (Fig. 2.31).

The liver can be better spotted in the left view of the body cavity. However, the stomach and the pyloric ceca are not very visible because the circumvolutions of the intestine overlap these structures (Figs. 2.32 and 2.33). The topography of internal organs of hybrid *Colossoma macropomum* × *P. mesopotamicus* is very similar to that of parental species (Ferreira et al., 2013).

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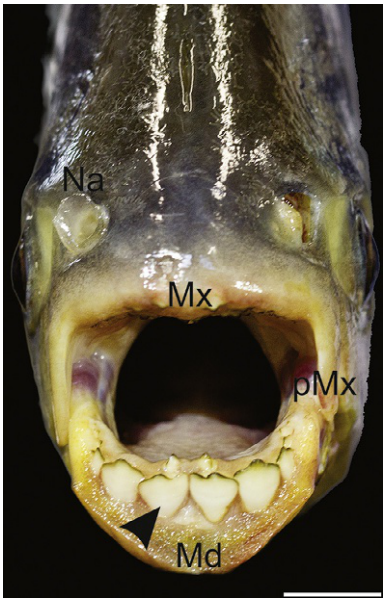

FIG. 2.28 Dentition of molariform aspect of pacu (*Piaractus mesopotamicus*), specialized in grinding hard foods, such as fruits and seeds. Molariform tooth (arrow), nostril (Na), mandible (Md), maxilla (Mx) premaxilla (pMx). Scale bar: 1 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

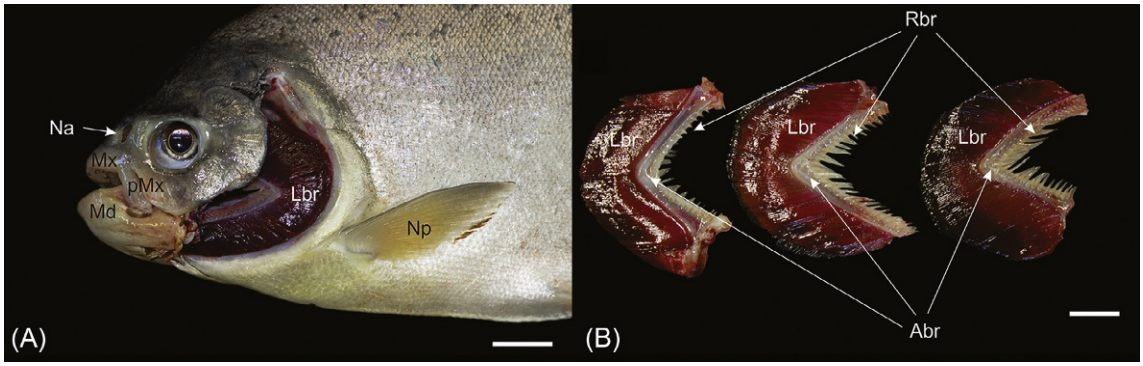

FIG. 2.29 Topographic location of the gills inside the orobranchial chamber (A) of pacu (*Piaractus mesopotamicus*). Detail (B) shows the branchial arches, which sustain the gill lamellae and the gills rakers. Gill lamellae (Lbr), gill rakers (Rbr), branchial arches (Abr), pectoral fin (Np), maxilla (Mx), premaxilla (pMx), mandible (Md), nostril (Na). Scale bar (A): 2 cm; (B): 1 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

Silver catfish *Rhamdia* sp.

The skin of the silver catfish (*Rhamdia* sp.) (Siluriformes: Heptapteridae) varies from reddish-brown to gray, with a lighter-colored ventral region (Gomes et al., 2000). The silver catfish has an elongated body, rounded near the head and compressed in the base region of a rather large adipose fin. The species has a depressed head and the mouth is in ventral position (Baumgartner et al., 2012) (Fig. 2.34).

As a rule, Siluriform catfish have sensory apparatus, the barbels, that are extensions of the integument inserted at the olfactory pits or nares (nasal barbels, one pair), at the maxillary region (maxillary barbels, one pair), and at the gular region (mandibular barbels, two pairs) (Diogo and Chardon, 2000; Schuingues et al., 2013). Barbels evolved to contain a high concentration of sensory structures, particularly taste buds, eliciting tactile and gustatory functions—the detection of food in the poorly lighted, murky bottom of the water body (Elliott, 2000; Schuingues et al., 2013) (Fig. 2.35). Catfish in general also present sensory structures, but in a lower number spread all through the surface of the body (Atema, 1971) (Fig. 2.35).

The silver catfish present a piscine muscle construct, similar to other teleost fish (Stiassny, 2000). However, due to the benthonic habit of most species, the muscle bundle corresponding to the *L. superficialis* does not have a dark coloration (myoglobin) (Fig. 2.36), which denotes a fish of low swimming activity compared to active swimmers such as the Characin "dourado" (*Salminus maxillosus*), for instance.

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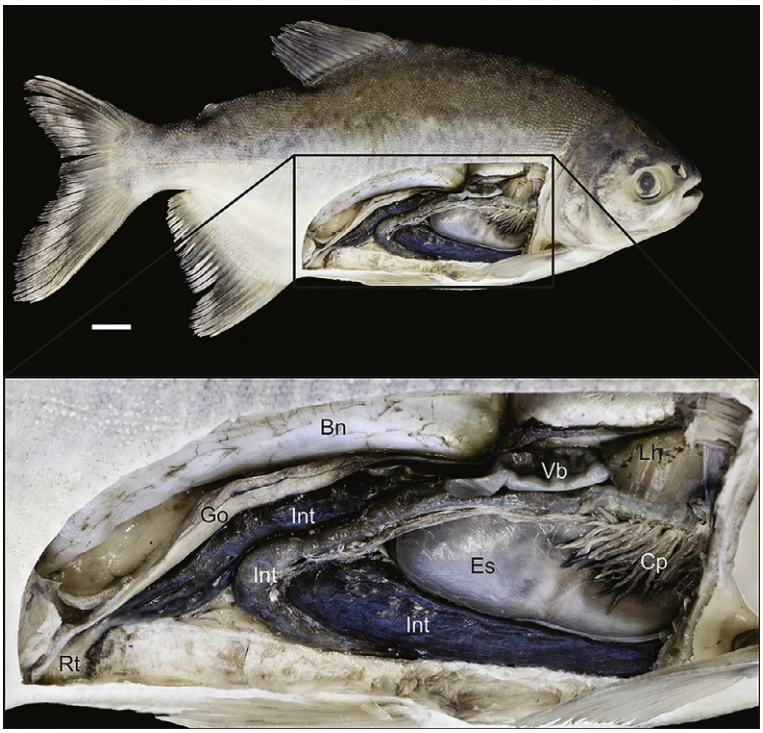

FIG. 2.30 Digestive system of pacu (*Piaractus mesopotamicus*). Esophagus (Esf), stomach (Es), pyloric caeca (Cp), cranial intestine (IntCr), caudal intestine (IntCd), and rectum (Rt). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

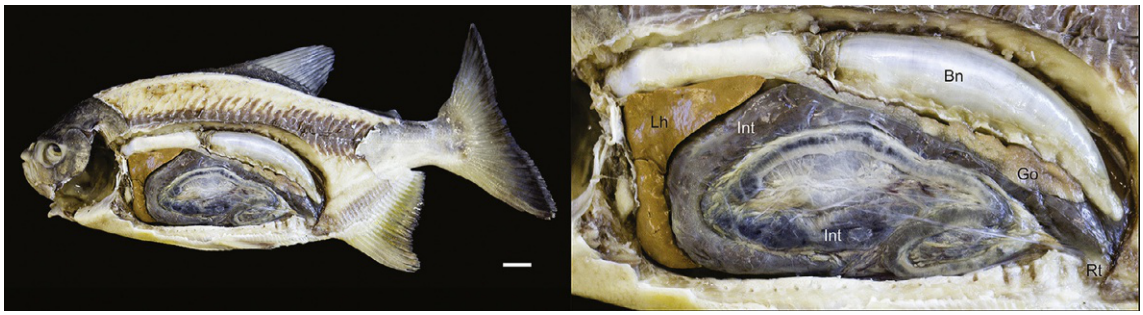

FIG. 2.31 Right side view of the internal organs of pacu (*Piaractus mesopotamicus*). In detail: swim bladder (Bn), gonads (Go), gallbladder (Vb), right hepatic lobe (Lh), stomach (Es), pyloric ceca (Cp), intestine (Int), and rectum (Rt). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

Accessing the visceral cavity of silver catfish from the ventral region allows spotting the corresponding organs of the digestive system, its annexes, and the reproductive system (Fig. 2.37). The swim bladder is located high in the visceral cavity, between the digestive tract and the kidney (Fig. 2.38). The catfish kidney is usually parted into a cephalic (cranial) and caudal portion. Some Siluriformes such as the silver and the channel catfish, *Ictalurus punctarus*, show a cephalic kidney as a separate structure from the caudal kidney, a detail described by Grizzle and Rogers (1976) (Fig. 2.39).

The silver catfish is an omnivorous species favoring small fish and crustaceans as the main food items. However, the anatomical features of its digestive tract are similar to those of carnivorous orichthyophagous fish: a simple gastrointestinal tract and the absence of a pyloric caeca or gizzard (Kütter et al., 2009) (Fig. 2.40).

Millet or pike cichlid *Crenicichla* sp.

As with any other cichlid fish, the pike cichlid *Crenicichla* sp. (Cichliformes: Cichlidae) is characterized by the preference for lentic environments. As a rule, cichlids provide parental care to eggs and early offspring, which guarantees reproductive success in reservoirs (Baumgartner et al., 2012). Cichlid fish show an interrupted lateral line split into an upper rostral branch and a lower hind branch, a mouth in terminal position, mobile premaxilla, conical teeth, a long dorsal fin with hard

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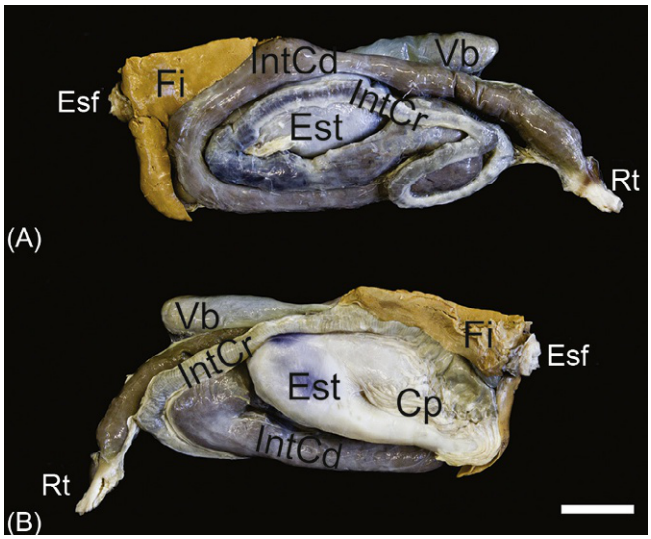

FIG. 2.32 Left side view of the internal organs of pacu (*Piaractus mesopotamicus*). In detail: swim bladder (Bn), gonads (Go), left hepatic lobe (Lh), intestine (Int), and rectum (Rt). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

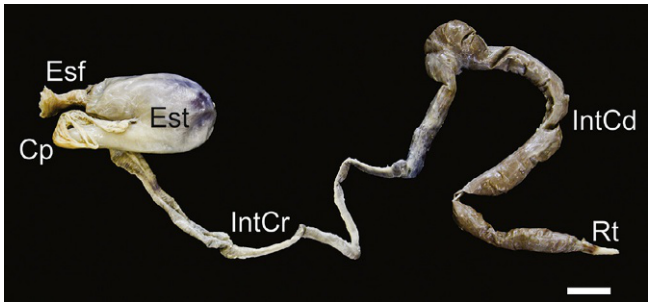

FIG. 2.33 Left (A) and right (B) side views of the set corresponding to the digestive system and annexed structures of pacu (*Piaractus mesopotamicus*). Liver (Fi), gallbladder (Vb), cranial intestine (IntCr), caudal intestine (IntCd), stomach (Est), esophagus (Esf), pyloric ceca (Cp), rectum (Rt). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

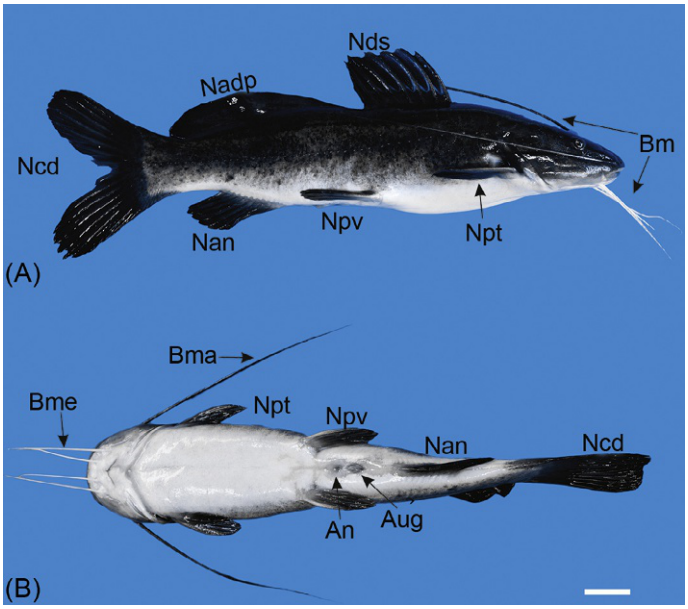

FIG. 2.34 Right (A) and ventral (B) side view of silver catfish (*Rhamdia* sp.). Mentonian barbels (Bm), pectoral fin (Npt), dorsal fin (Nds), pelvic fin (Npv), anal fin (Nan), adipose fin (Nadp), caudal fin (Ncd), anus (An), urogenital opening (Aug). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

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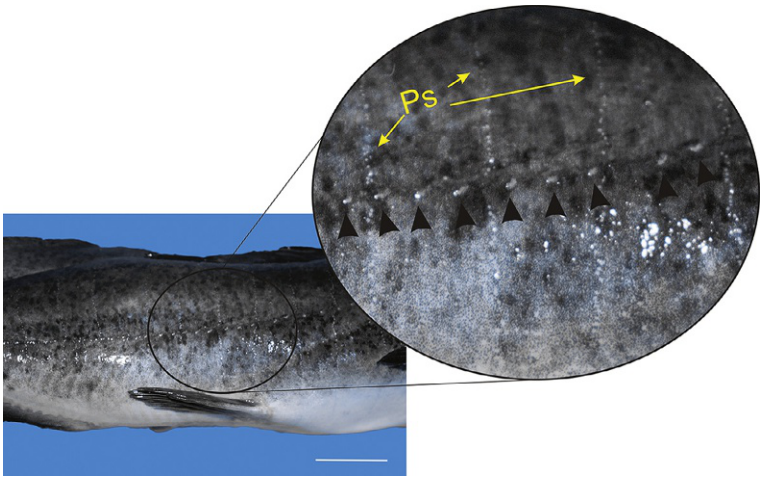

FIG. 2.35 Right side view of silver catfish (*Rhamdia* sp.). In detail: lateral line (▲) and sensory pores (Ps) in the surface of the fish, which has taste buds that help in locating the food. Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

FIG. 2.36 Right side view of silver catfish (*Rhamdia* sp.) muscle tissue and delimitation of different anatomical regions. Epaxial myomere (mEpx), hypaxial myomere (mHpx), *Lateralis superficialis* muscle (mLsp). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

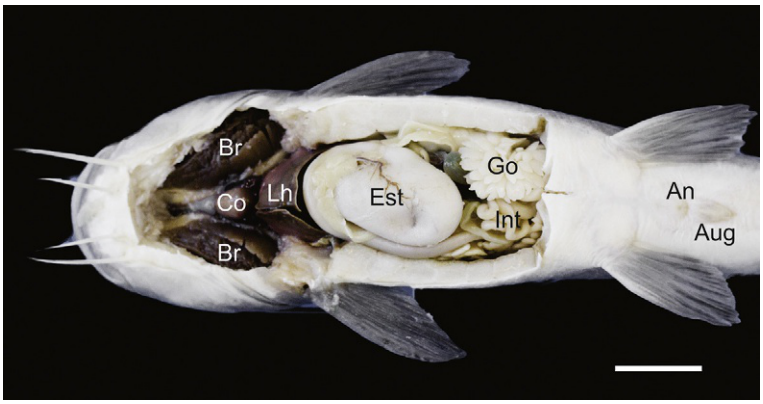

FIG. 2.37 Central view of the internal organs of silver catfish (*Rhamdia* sp.). Gills (Br), heart (Co), hepatic lobes (Lh), stomach (Es), intestine (Int), gonads/testis (Go), anus (An) and urogenital opening (Aug). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

FIG. 2.38 Central view of the visceral cavity of silver catfish (*Rhamdia* sp.) after removal of the digestive and reproductive systems. Swim bladder (Bn), cephalic (Rcf) and caudal (Rcd) region of the kidney. Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

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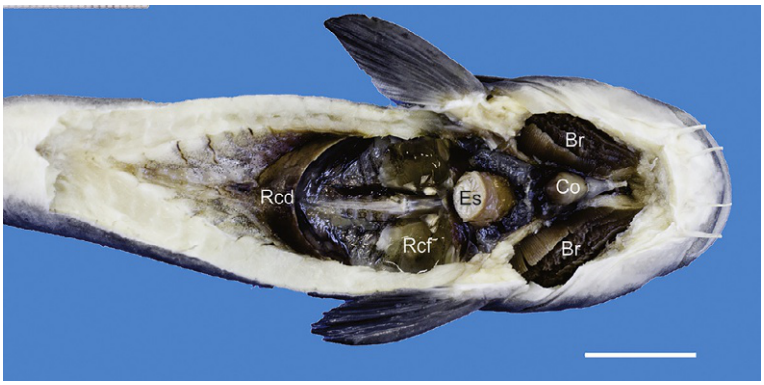

FIG. 2.39 The kidney of silver catfish (*Rhamdia* sp.). Cephalic region separated from the caudal one. Gills (Br), heart (Co), esophagus (Esf), cephalic (Rcf) and caudal (Rcd) region of the kidney. Scale bar: 2.5 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

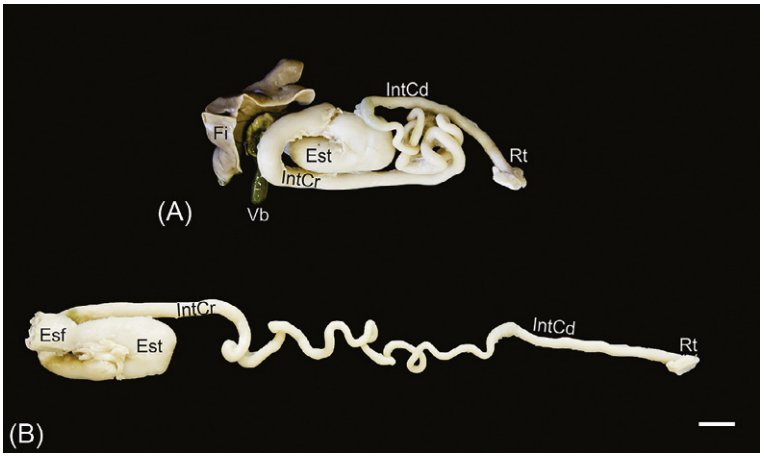

FIG. 2.40 Left side view of silver catfish (*Rhamdia* sp.) digestive system with annexed structures (A), and simple gastrointestinal tract (B) without annexed structures. Liver (Fi), gallbladder (Vb), cranial intestine (IntCr), caudal intestine (IntCd), stomach (Est), esophagus (Esf), rectum (Rt). Scale bar: 1 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

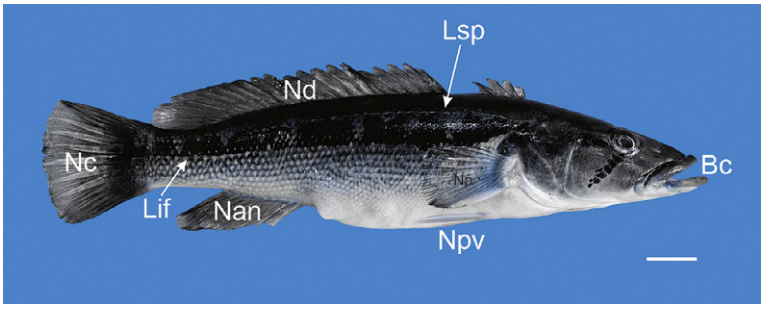

FIG. 2.41 Right side view of millet or pike cichlid (*Crenicichla* sp.). Mouth (Bc), pectoral fin (Np), dorsal fin (Nd), pelvic fin (Npv), anal fin (Nan), caudal fin (Nc), upper (Lsp), and lower (Lif) lateral line. Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

spines in the cranial region, and soft spines in the caudal region (Kullander, 2003; Varella et al., 2018) (Fig. 2.41). The long maxilla and projected and protractile mandible with small, conical teeth indicate the capacity of fish from this genus to capture their food (prey) in the water column (Sampaio and Goulart, 2011) (Fig. 2.42). The central view of the visceral cavity with the topography of the viscera is shown in Fig. 2.43.

The digestive tract of the millet is similar to that of neotropical cichlids such as the *Satanoperca pappaterra*, which has a sacciform, a small stomach at the anterior position (Hahn and Cunha, 2005), indicating that the organ elicits only short-term food passage (Sampaio and Goulart, 2011); the liver of millets have long hepatic lobes that, caudally oriented (Fig. 2.44). The intestine of *Crenicichla* sp. is considered relatively short compared to other neotropical cichlids, especially those with an omnivorous feeding habit (Sampaio and Goulart, 2011).

Fish of carnivorous and ichthyophagous feeding habits have short intestines resulting from the low content of lignin, an item of difficult digestion, in their food. This explains in part the shorter intestine of the millet or pike cichlid, whose smaller specimens feed on aquatic insects (Diptera, Ephemeroptera, Odonata etc.) and whose larger specimens feed almost

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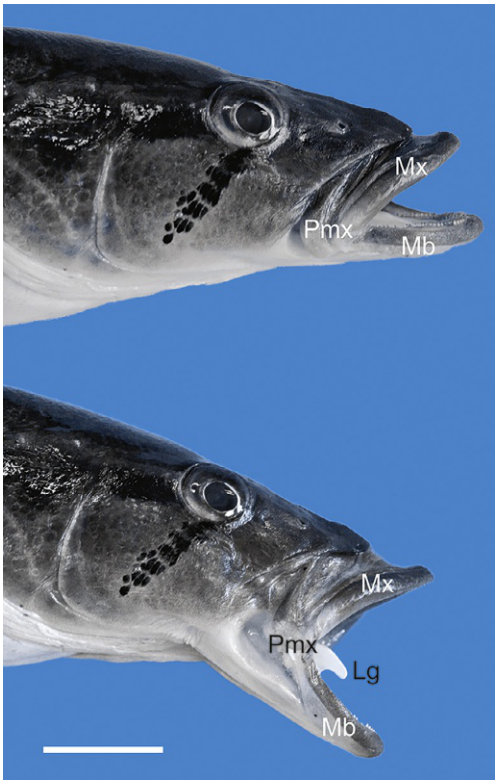

FIG. 2.42 Detail of the protractile mouth of millet or pike cichlid (*Crenicichla* sp.). Premaxilla (PMx), maxilla (Mx), mandible (Mb), tongue (Lg). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

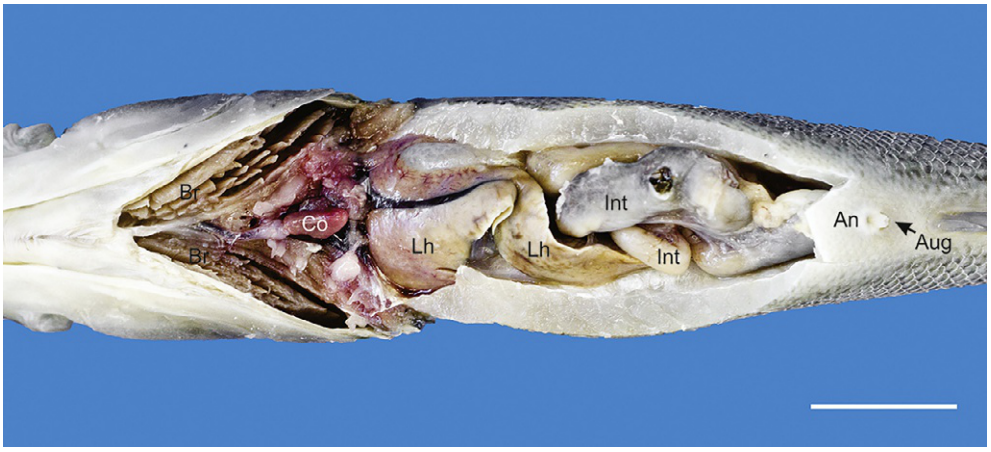

FIG. 2.43 Ventral view of the internal organs of millet or pike cichlid (*Crenicichla* sp.). Gills (Br), heart (Co), hepatic lobes (Lh), intestine (Int), anus (An) and urogenital opening (Aug). Scale bar: 2.5 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

exclusively on fish (Montaña and Winemiller, 2009) (Fig. 2.45). Finally, the swim bladders of millets are dorsally located between the viscera and the kidney (Fig. 2.46).

Final considerations

The morphology and anatomy of the different systems of fish are still underexplored in neotropical Teleosts and cartilaginous fish. Therefore, a vast opportunity for the acquisition of new knowledge on this topic is present, given the diversity and large number of species that constitute the neotropical ichthyofauna. This subject is important not only for systematics and taxonomic purposes, but also for physiological studies, mainly for the species of interest for aquaculture in the neotropics, bringing tools for a better handling and productive performance.

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FIG. 2.44 Detail of the digestive tract of millet or pike cichlid (*Crenicichla* sp.). There is a liver with long hepatic lobe that is caudally directed and a small sacciform stomach in the anterior region. Liver (Fi), stomach (Est), esophagus (Esf) and cranial intestine (IntCr). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

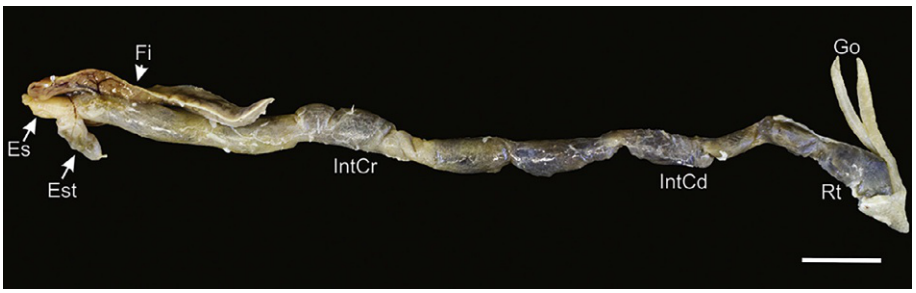

FIG. 2.45 Digestive tract and gonads of millet or pike cichlid (*Crenicichla* sp.). Sacciform stomach in the anterior region and relatively small intestine compared the omnivorous species of neotropical cichlids. Liver (Fi), stomach (Est), esophagus (Esf), cranial (IntCr) and caudal (IntCd) intestine, rectum (Rt) and gonads (Go). Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

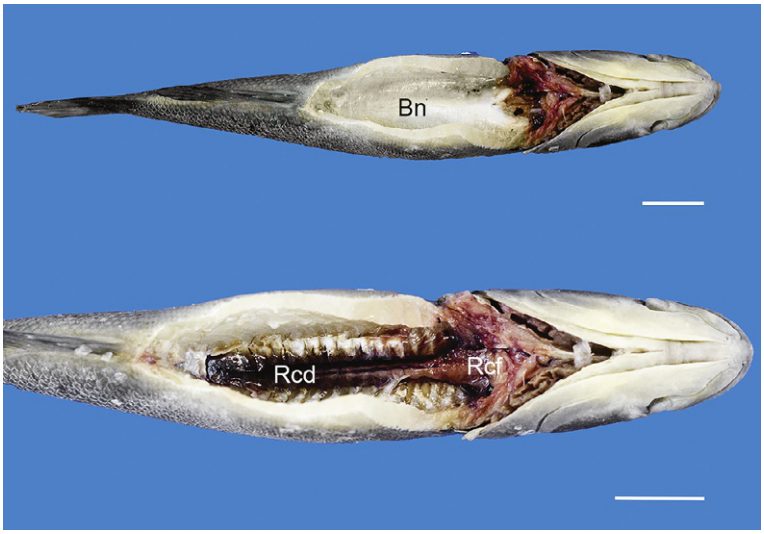

FIG. 2.46 Ventral view of millet or pike cichlid (*Crenicichla* sp.). Swim bladder located between viscera and kidney. Swim bladder (Bn), cephalic (Rcf) and caudal (Rcd) region of the kidney. Scale bar: 2 cm. *(Courtesy: Carolina Zabini. Reprinted with permission of the author.)*

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Chapter 3

The genetic bases of physiological processes in fish

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Introduction

In early studies of physiological processes, French physician and physiologist Claude Bernard (1813–78) coined the phrase *milieu intérieur* when he wrote that "the stability of the internal environment [the *milieu intérieur*] is the condition for the free and independent life." Based upon that concept, American physiologist Walter Bradford Cannon (1871–1945) coined the concept of homeostasis (Cannon, 1932), under which living beings have developed mechanisms to keep their physiological parameters within strict limits. However, Russian physiologist Mrosovsky (1990) reexamined the concept of homeostasis in his book "*Rheostasis: The Physiology of Change*," in which he posited that instead of maintaining the constancy of their internal environment or having effective mechanisms to preclude changes, living organisms exhibit physiological mechanisms that cope with changes in regulated levels, which those organisms use to face environmental changes. Mrosovsky termed those mechanisms "rheostasis."

Fish are singular examples of how changes in environmental factors operate throughout a living organism's life cycle. For instance, aquatic organisms must cope with differences in temperature, oxygen concentration, water osmolarity, and other environmental factors all through their lives. Therefore, it is important to understand how aquatic animals attain allostasis, the process whereby internal stability—that is, homeostasis—through physiological or behavioral change is maintained, or adapt to the (environmental) changes through rheostasis (Wikelski and Cooke, 2006). The basis for triggering these physiological processes is encoded in the genetic makeup of populations. Dobzhansky and Wallace (1953) elegantly expressed the importance of genetic variability to physiological processes, stating *verbatim* that "Adaptation to a variety of environments is accomplished in two ways. First, most species and populations are polymorphic and consist of a variety of genotypes optimally adapted to different aspects and sequences of environments. Secondly, individuals respond to environmental changes by physiological and structural modifications. Modifications evoked by environmental variations recurrent in the environment of the species almost always tend to increase the probability of survival and reproduction of the organism. The organism adjusts itself to recurrent environmental changes in such a way that its functioning continues unimpaired; it is said to be homeostatic."

The core of knowledge on the physiology of Neotropical fish was positively impacted by contributions from the Brazilian scientist Dr. Carlos Chagas Filho, who studied electrogenesis in the electric eel, *Electrophorus electricus* (Albe-Fessard et al., 1951; Chagas et al., 1953). Brazilian zoologist and biologist Dr. Rodolpho T.W.G. von Ihering studied fish reproduction and set the basis for the reproductive manipulation of Neotropical rheophilic fish, that is, fish living in

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flowing waters. At the International Congress of Physiology in the former USSR in 1935, Ihering and colleagues presented studies that demonstrated complete control of the spawning process of rheophilic fish by injecting pituitary extract (von Ihering et al., 1935; von Ihering and Azevedo, 1936; von Ihering, 1937). Artificial propagation allowed the development of the aquaculture of migratory Neotropical species, such as the characins pacu *Piaractus mesopotamicus*, tambaqui *Colossoma macropomum*, the siluriform surubim *Pseudoplatystoma corruscans*, and many other species, allowing mass fingerling production in confinement.

The seminal advance in genetics was the elucidation of base pairing and the double-helix structure of DNA by Watson and Crick (1953), a milestone enabling the understanding of how genes encode proteins and control physiological processes from the cell to the whole organism level. During the 50 years following Watson and Crick's landmark publication, the previously separate fields—physiology and genetics—converged to elucidate the pathways underlying the regulation of gene expression, homeostasis, organism-environment interactions, and the determination of phenotype.

Fish are extraordinary vertebrate models for integrative studies of genetics and physiology. Freshwater fish inhabit diverse ecosystems and habitats, from streams at high elevation to lakes, floodplains, and rivers running through different latitudes and climatic zones to the depths of ocean trenches. Ray-finned fish comprise approximately half the diversity of all vertebrates, with about 33,000 named species (Nelson, 2006; Froese and Pauly, 2018); remarkably, around 15,000 species live in freshwater environments (IUCN, 2018). In Neotropical freshwater ecosystems, ichthyodiversity accounts for >7000 species, with a significant number that are important as genetic resources supporting regional nutrition and income (Hilsdorf and Hallerman, 2017). The capacity of physiological adaptation that Neotropical fish have developed through evolution is shown across the many different environments throughout the Amazon watershed. These environments harbor striking physical (including water quality), chemical, and biological heterogeneity with a wide range of morphological and physiological adaptations driving the impressive diversity of freshwater fish (Val et al., 1996).

Diverse topics on the physiology of Neotropical fish are yet to be investigated. For instance, the physiological genomic mechanisms underlying the rheophilic reproductive cycle in the wild—and in confinement—are poorly understood. What are the genes involved? What are the environmental triggers, and how are they transduced into gene expression and physiological adaptation? Better understanding of the interplay of genetic and physiological processes can be applied to understand adaptation at a mechanistic level, to genetically improve rheophilic species for aquaculture, and to better sustain management of wild populations impacted by anthropogenic disturbances such as overfishing, pollution, and damming of rivers for hydropower generation.

Fish have been an important source of food throughout human history, first from fisheries and later from aquaculture. The current scenario has not changed, except for the massive scale of capture from commercial fisheries (Worm et al., 2006), the growth of world aquaculture (FAO, 2016), and in some areas of aquaculture, the increase in productivity from the use of superior, selectively bred strains (Gjedrem and Robinson, 2014). The genetic and physiological processes underlying fishery-induced evolution (the adaptive responses of wild fish stocks under constant fishing pressure) and the adaptation of cultured stocks to aquaculture (Hutchings and Fraser, 2008) are still to be fully unraveled. In recent years, novel molecular technologies, particularly next-generation sequencing (NGS), have hastened advances in genetic and physiological studies of nonmodel organisms, unlocking the organismal "black box" to address the role of physiology in the mechanisms translating genetic into phenotypic variation. This chapter introduces and discusses linkages between genetics and physiology as applied to fish farming and genetic resource conservation, first by addressing the importance of genetic diversity to the management of wild populations and the domestication and genetic improvement of aquaculture species. That is followed by a discussion on how new molecular technologies can improve the understanding and management of genetic resources of finfish species as well as the molecular bases of the fish reproductive cycle, mainly concerning rheophilic species. Finally, we explore chromosome set manipulation and gene transfer technologies as tools for investigating physiological processes in fish.

Genetic diversity and physiological adaptation

The Earth harbors an amazing diversity of species, the result of continuing ecological adaptation and evolution. Adaptation acts upon heritable genetic variation within a species, with variants enabling high fitness being transmitted from one generation to the next. Changes in allele frequencies through time may result in altered phenotypes as well as novel traits. Populations differentiate from one another through the combined action of the processes of mutation, migration, selection, and random genetic drift. Speciation occurs through the dynamic interplay of geographic dispersal and isolation, ecological adaptation, and development of reproductive isolation mechanisms (Mayr, 1963).

Fish provide extraordinary examples of diverse speciation mechanisms (Seehausen and Wagner, 2014). Aquatic ecosystems are highly variable through space and time, showing dramatic gradients of ecological conditions to which fish must cope and adapt. Natural or anthropogenic-driven variations in temperature, pH, ion concentration, salinity, and oxygen, for

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instance, characterize aquatic environments. These abiotic factors may lead to diverse physiological responses within and among genetically adapted populations. Not only is the Neotropical region especially subject to marked environmental oscillations (Peçanha et al., 2017), but it is also common to find distinct aquatic habitats subject to different hydromorphological processes in contiguous Neotropical areas (Junk et al., 2014). For instance, the Pantanal floodplain, located in a wide, shallow depression in central South America in the upper Paraguay River basin of Brazil, is one of the largest and most important wetland ecosystems in the world (Junk and Cunha, 2005). This vast ecosystem is characterized by an annual flooding regime, characterized by flood pulsing. Such a dynamic creates a myriad of habitats deeply affecting the local biodiversity, particularly fish assemblages (Alho, 2008). Just north of the Pantanal floodplain, the Amazon basin emerges. Despite the proximity of these ecosystems, the Amazon River and its tributaries are subject to hydrodynamics and water conditions distinct from those in the adjacent Paraguay basin. For instance, fish dwelling in Amazonian waters must adapt to dissolved oxygen availability ranging from zero at night to saturation during daylight. These extreme oxygen variations demand physiological adjustments of local fish species, which are pivotal to their adaptation and survival (Val and Almeida-Val, 1999).

The ability of a single genotype to produce multiple phenotypes in response to changes in environmental conditions is known as "phenotypic plasticity" (Pigliucci, 1996; Iwama et al., 1998; Kelly et al., 2012). The phenotypic plasticity extant in plants and animals, particularly freshwater fish, sets up an interface between genetic variation and the potential for adaptation to environmental changes (Walker, 1997). The adaptive value represents a quantitative measure of the fitness of an individual, determining its likelihood of survival to reproductive age and reproduction to transmit its genotype to the next generation. The genetic diversity within and among populations thus becomes a requirement for the development of locally adapted phenotypes. This genetic diversity is measured by several metrics, such as the fraction of individuals in a population that are heterozygous at a particular locus (heterozygosity), the number of alleles per locus (allelic diversity), and the proportion of polymorphic loci across the genome (gene diversity). Consequently, there is an expected positive correlation between fitness and genetic diversity at the population level, which supports the view that loss of heterozygosity through inbreeding directly affects population-level fitness (Mitton and Grant, 1984; Allendorf and Leary, 1986; Reed and Frankham, 2003). In addition, loss of genetic variation as a result of random genetic drift impacts the fitness of a population. For instance, Wang et al. (2002) showed the positive correlation between genetic variability and fitness among salmonids, important to both fisheries and aquaculture. Whereas this study showed that this correlation varies and can be tenuous, yet disease resistance, growth rate, fecundity, physiological efficiency, and other fitness traits may be affected by the genetic makeup at the individual and population levels.

On the other hand, even small populations with depleted genetic diversity can retain phenotypic plasticity through the expression of epigenetic variation, a nongenetic source of phenotypic variation (Francis, 2011; Verhoeven et al., 2016). Studies on epimutations carried out with fish have shown the expression of physiological phenotypes under environmental stressors during the organism's life cycle (Baerwald et al., 2016; Smith et al., 2016; Best et al., 2018; Labbé et al., 2017) as well as the effect of epimutations on aquaculture productivity (Moghadam et al., 2015). The Amazon basin, with its diverse ichthyofauna and myriad environmental conditions, is an open laboratory for unraveling the putative epigenetic mechanisms underlying physiological adaptations of Amazon fish. A vast array of scientific literature has demonstrated the adaptation of the Amazon fish to the river's environmental modifications (Almeida-Val et al., 1993; Val et al., 1998; Saint-Paul and Soares, 1998; Aride et al., 2007; Lewis et al., 2007; Val et al., 2016; Jesus et al., 2017b).

Fisheries and aquaculture are important sources of food and income worldwide. According to the FAO (2016): "World *per capita* fish supply reached a new record high of 20 kg in 2014, thanks to vigorous growth in aquaculture, which now provides half of all fish for human consumption, and to a slight improvement in the state of certain fish stocks due to improved fisheries management. Moreover, fish continues to be one of the most-traded food commodities worldwide, with more than half of fish exports by value originating in developing countries." Therefore, the conservation and sustainable management of aquatic genetic resources (AqGR) is essential for sustaining long-term protein production from fish in the face of an ever-increasing human population. AqGR supports productivity and contributes to the long-term viability of fisheries and aquaculture production.

The Neotropical region harbors the world's most diverse AqGR. The importance of assessment and management of these resources was reviewed recently by Hilsdorf and Hallerman (2017). It was only in the 1960s that pioneering attempts to characterize fish population differentiation using electrophoresis and histochemical staining techniques to observe the activities of protein-encoding allozyme loci were made (Whitmore, 1990). In the early 1980s, variation of mitochondrial DNA was characterized to investigate matrilineal genetic structure in fish (as reviewed by Avise, 2004). A turning point in the use of DNA markers in population genetic studies was the development of the polymerase chain reaction, or PCR (Saiki et al., 1988), which allowed the targeted amplification of a DNA sequence of interest and its characterization. PCR made possible the rapid development of tandemly repeated microsatellite DNA markers for a large number of species.

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Microsatellites are arrays of tandem repeats of short DNA motifs; the numbers of such repeats at a locus may vary among individuals in a population and among populations. Microsatellites have become a powerful genetic marker for many different applications, such as population genetics, linkage mapping, and quantitative trait locus detection, among others (Chistiakov et al., 2006). More recently, single nucleotide polymorphism (SNP) has emerged as a marker of choice given the variety of applications in genetic studies (Fig. 3.1). SNPs were first characterized in humans (Sachidanandam et al., 2001) as the results of single mutation events that occur at specific positions in the genome at a frequency of >1% within a population. The utility of SNPs as codominant and high-density DNA markers has driven their identification and use in both model and nonmodel animals and plants (Vignal et al., 2002; Mammadov et al., 2012; Wenne, 2018).

Assessment of genetic differentiation among populations may reveal mechanisms of physiological adaptation. The Neotropical region encompasses innumerable environments and a vast diversity of life forms. That is particularly true for freshwater ichthyodiversity, with the majority of freshwater fish inhabiting the Neotropics (Reis et al., 2016). According to Hilsdorf and Hallerman (2017), the degree of population genetic differentiation (quantified by F_{ST}-like and $F_{ST\text{-adjusted}}$ metrics) ranges from moderate to great across the different Neotropical freshwater fish orders. Genotypic local adaptations of populations play a fundamental role in the evolution of a given species as a whole (Blanquart et al., 2013). Particularly for commercially important species, local adaptations are the raw genetic material on which food production relies, first in order to yield better crop, livestock, forestry, and fish production in the present, and second, because of the paramount importance for food security, currently and into the future. This raw genetic material may represent differences in patterns of gene expression for traits underlying local adaptation. Previous studies using single-gene expression methodologies involving candidate genes, or even genome scans for quantitative trait measurements, have supported that view. For example, Partridge et al. (2004) assessed differences in growth rate between populations of the economically important marine fish, black bream, *Acanthopagrus butcheri*. They demonstrated that juveniles originating from two reproductively isolated river estuaries had substantial differences in growth rate, inferring differential local adaptation of different sets of genes to the respective estuarine conditions. Picard and Schulte (2004), using differential display PCR, identified differences in expression of hepatic genes responsive to handling stress among populations of common mummichog, *Fundulus heteroclitus*. Larsen et al. (2012) used quantitative RT-PCR assays for expression of the heat shock protein *hsp70* and the *Na^+,K^+-ATPase-α* genes to demonstrate different responses to salinity conditions among two populations of Atlantic cod, *Gadus morhua*. These outcomes suggest local adaptation of populations, even under low levels of genetic divergence observed at selectively neutral molecular markers.

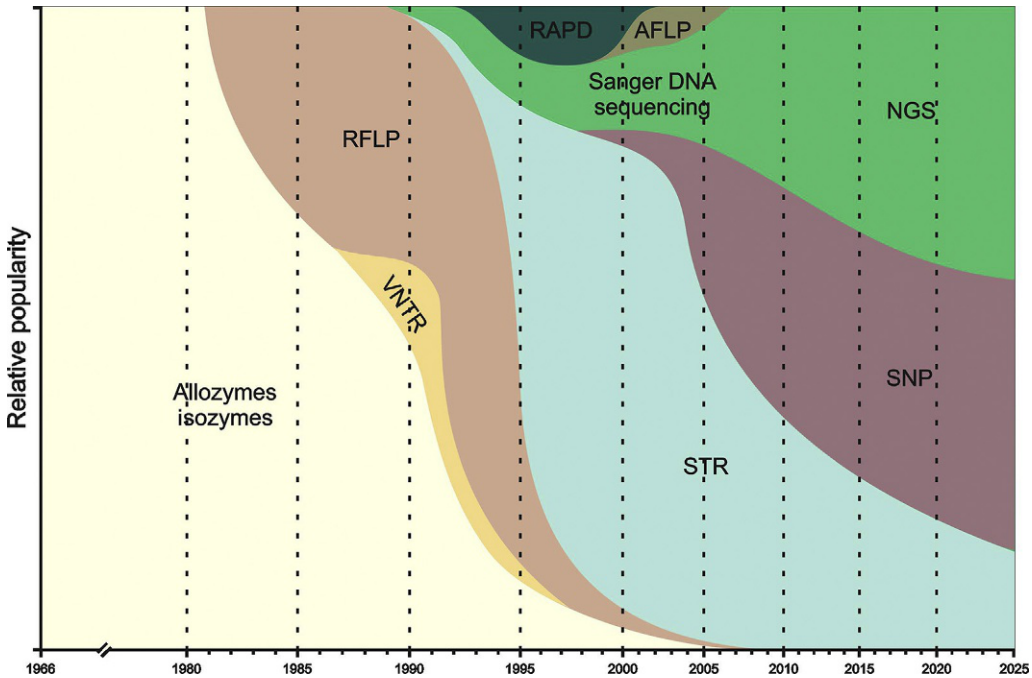

FIG. 3.1 The changing relative importance of different genotyping strategies through time (see page 65 in Hilsdorf and Hallerman, 2017). Abbreviations: *RFLP*, restriction fragment amplify polymorphism; *RAPD*, random amplification of polymorphic DNA; *AFLP*, amplified fragment length polymorphisms; *VNTR*, variable number of tandem repeats (minisatellites); *STR*, short tandem repeats (microsatellites); *NGS*, next generation sequencing; and *SNP*, single nucleotide polymorphism.

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Over the past decade, a revolution in DNA sequencing, known as NGS, has been accomplished (Goodwin et al., 2016). Several sequencing platforms were developed and became readily available, routinely yielding sizable amounts of data in a single instrument run (Shendure and Ji, 2008; Levy and Myers, 2016). Whole-genome, transcriptome, and interactome sequences of nonmodel organisms are currently at hand for researchers, providing affordable results with less production-scale effort. The power of genome-scale sequencing is echoed by the fact that, currently, there are >50 species of ray-finned fish (Class Actinopterygii) genomics projects ongoing at different stages of assembly, mostly involving freshwater species (Spaink et al., 2013; Yuan et al., 2018). Some of them are key for aquaculture, such as Nile tilapia *Oreochromis niloticus*, channel catfish *Ictalurus punctatus*, and common carp *Cyprinus carpio*.

The advent of NGS facilitated the development of high-throughput methodologies relying on the proprieties of restriction enzymes to enable low-cost discovery and genotyping of high numbers of polymorphic markers for any organism. The different methods, depending on the genomic library preparation protocols, are termed original restriction site-associated DNA sequencing (RADseq), and variants such as double-digest RADseq (ddRADseq), IIB restriction enzymes RADseq (2bRADseq), and genotyping by sequencing (GBS). For a thorough review of the use and applications of such powerful genetic marker discovery methods, see Andrews et al. (2016). Among other applications, these NGS-based methods for SNP discovery boosted the development of high-density genetic linkage maps for diverse aquaculture and fisheries species (Table 3.1).

Construction of genetic linkage maps is a required step for understanding the genome organization, whereby comparative studies can be implemented to delve into the genetic basis of genome structure, patterns of gene expression, gene function, physiological adaptation, and evolution (Kumar and Kocour, 2017). Aquaculture and fisheries management have taken advantage of the use of the massive amount of sequence data of fish and other aquatic species (Robledo et al., 2017). SNP arrays, that is, a specific type of DNA microarrays, have been developed for several aquaculture species (Table 3.2). Their utilization will reduce the costs of scoring large numbers of SNPs.

Application of genomic technology is narrowing the gap between understanding genetic variation (genotype) and physiology (phenotype). Classical understanding of population genetic structure based on the variation of a small collection of neutral genetic markers is moving toward a genome-wide level of understanding based on the variation at thousands of SNPs, in which variation at the gene level may be directly associated with fitness, adaptation to environmental changes, expression of inbreeding depression, and processes of speciation. In particular, the application of genomic approaches may enable elucidation of the molecular variation underlying mechanisms of local adaptation of wild and aquacultured genetic resources of fundamental importance for long-term food production (Wambugu et al., 2018; Kantanen et al., 2015; Hansen et al., 2012). According to Nielsen and Pavey (2010), genomic assessments of fish populations can be grouped into three broad categories: (i) evolutionary genomics and biodiversity; (ii) adaptive physiological responses to a changing environment; and (iii) adaptive behavioral genomics and life-history diversity.

In this context, population genomics emerges as a new discipline. Black et al. (2001) introduced the term "population genomics" as the "process of simultaneous sampling of numerous variable loci within a genome and the inference of locus-specific effects from the sample distributions." Population geneticists now can differentiate locus-specific effects from genome-wide effects. Use of NGS-associated methodologies yields whole-genome markers to detect the signature of local adaption, from which new theoretical understandings can be reached and new analytic approaches developed (Hoffmann and Willi, 2008; Bernatchez, 2016). Under local directional selection, locus-specific adaptive variation behaves differently than variation at selectively neutral loci, revealing "F_{ST} outlier" loci, whereby genes underlying fitness and adaptation exhibit high levels of differentiation among populations and can thereby be identified (Luikart et al., 2003). To do so, the landscape genomics approach assesses geographic patterns of population distribution associated with genome-wide variation in order to identify genes that underlie fitness and local adaptation (Sork et al., 2013) (Fig. 3.2).

As an example of where the application of population genomics may yet enable insights into adaptation, a return to the molecular genetic studies of fish native to the Pantanal is opportune. Fish of the Pantanal ecosystem have shown a lack of population genetic structuring that is generally explained by the seasonal flooding dynamics of that ecosystem, which promotes mixing of juveniles from distinct rivers, and as a result, panmixia (Calcagnotto and DeSalle, 2009; Iervolino et al., 2010; Mondin et al., 2018). For instance, Okasaki et al. (2017) screened microsatellite DNA markers and found no genetic structuring among populations of *Brycon hillari* (Characiformes) from four rivers. However, low but significant genetic differentiation was noted between populations of the Taquari River and the Cuiabá and Paraguay rivers. The Taquari River is characterized as a megafan river (Assine, 2005) where the seasonal flooding process causes avulsion, altering the Taquari riverbed and often shifting its course. The mechanism by which megafan stream dynamics may promote population divergence (and even speciation; Wilkinson et al., 2006) may partially explain the significant genetic divergence and relatively high number of private alleles in the *Brycon hilarii* population inhabiting the Taquari River. Application of new genomic approaches might lead to the discovery of F_{ST} outlier loci in many Neotropical fish and may reveal patterns of local

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TABLE 3.1 Genetic linkage maps developed for aquaculture species

Species	Number of SNPs	Number of linkage groups	Map length, centiMorgans	References
Atlantic cod (*Gadus morhua*)	4753	23	1421.92	Hubert et al. (2010)
Atlantic salmon (*Salmo salar*)	5560	29	2403 (female) 1752.9 (male)	Lien et al. (2011)
Rainbow trout (*Oncorhynchus mykiss*)	2226	29	3600	Guyomard et al. (2012)
Atlantic halibut (*Hippoglossus hippoglossus*)	5703	24	1496 (female) 1378.1 (male)	Palaiokostas et al. (2013)
Atlantic salmon (*Salmo salar*)	6000	29	1426 (female) 2358 (male)	Gonen et al. (2014)
Chinook salmon (*Oncorhynchus tshawytscha*)	6352	34	4163.9	Brieuc et al. (2014)
Japanese eel (*Anguilla japonica*)	2672	19	1748.8 (female) 1294.5 (male)	Kai et al. (2014)
Japanese flounder (*Paralichthys olivaceus*)	13,362	24	3497.29	Shao et al. (2015)
Asian seabass (*Lates calcarifer*)	3321	24	1577.67	Wang et al. (2015a)
Turbot (*Scophthalmus maximus*)	6647	22	2622.09	Wang et al. (2015b)
Japanese amberjack (*Seriola quinqueradiata*)	9356	24	1029.24 (female) 1227.95 (male)	Aoki et al. (2015)
European sea bass (*Dicentrarchus labrax*)	6706	24	4816.93	Palaiokostas et al. (2015)
Large yellow croaker (*Larimichthys crocea*)	10,150	24	5451.3	Ao et al. (2015)
Large yellow croaker (*Larimichthys crocea*)	3448	24	2632	Xiao et al. (2015)
Channel catfish (*Ictalurus punctatus*)	54,342	29	3505.4	Li et al. (2015)
Bighead carp (*Hypophthalmichthys nobilis*)	3121	24	2341.27	Fu et al. (2016)
Common carp (*Cyprinus carpio*)	28,194	50	10,595.94	Peng et al. (2016)
Atlantic salmon (*Salmo salar*)	96,000	29	7153.2 (female) 4769 (male)	Tsai et al. (2016)
Arctic charr (*Salvelinus alpinus*)	4508	39	4302.7 (female) 2808.5 (male)	Nugent et al. (2017)
Blunt snout bream (*Megalobrama amblycephala*)	14,648	24	3258.38	Wan et al. (2017)
Mandarin fish (*Siniperca chuatsi*)	3283	24	1972.01	Sun et al. (2017)
Tambaqui (*Colossoma macropomum*)	7734	27	2811	Nunes et al. (2017)
Southern flounder (*Paralichthys lethostigma*)	2847	24	1605.43	O'Leary et al. (2018)

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TABLE 3.2 Development of high-density SNP BeadChip arrays in aquaculture species (FAO, 2017)

Species	SNP array technology	SNP array density	References
Atlantic salmon	Illumina iSelect	15 K	Gidskehaug et al. (2011)
Atlantic salmon	Affymetrix Axiom	286 K	Houston et al. (2014)
Channel catfish	Affymetrix Axiom	250 K	Liu et al. (2014)
Channel catfish	Affymetrix Axiom	690 K	Zeng et al. (2017)
Common carp	Affymetrix Axiom	250 K	Xu et al. (2014)
Rainbow trout	Affymetrix Axiom	57 K	Palti et al. (2015)

adaptations not unveiled by screening selectively neutral DNA markers, a potentially important research line. In other words, the detection of distinct local populations with signatures of genomic adaption may lead to better-informed fishery management plans, and possibly to the establishment of new protected areas.

Studies of fish genomics, particularly of economically important species, have provided evidence of the signatures of long-term natural and artificial selection. Many wild fish stocks in fresh and marine waters have been under strong fishing pressure, especially after the 1950s and 1960s when advanced fishing technologies were implemented (Eigaard et al., 2014). The relentless harvest that followed not only reduced stock sizes, but also led to larger fish being harvested as a result of size-selective fishing (Conover and Munch, 2002; Walsh et al., 2006; Dunlop et al., 2009). Some questions remain: (i) what are the genetic variants under selection?, (ii) what are the physiological consequences concerning the life cycle of

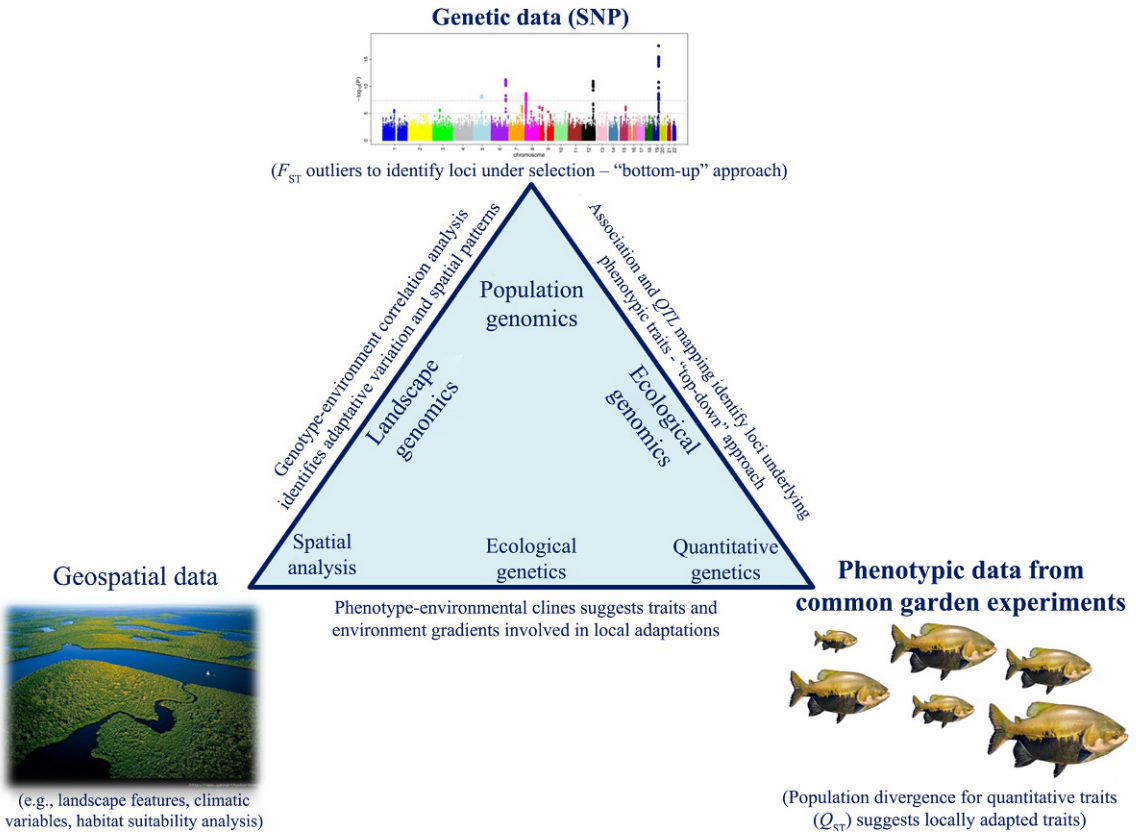

FIG. 3.2 Assessment of the interactions among phenotypic, genomic, and geospatial data to identify local adaptative genetic variation. *(Adapted from Sork, V. L., Aitken, S. N., Dyer, R. J., Eckert, A. J., Legendre, P., Neale, D. B. 2013. Putting the landscape into the genomics of trees: approaches for understanding local adaptation and population responses to changing climate*. Tree Genetics and Genomics *9, 901–911).*

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these stocks?, and (iii) can populations recover from size-selective fishing? Solving these questions is essential for the long-term sustainable use or even the sustainability of the AqGRs.

In another context, methodologies associated with NGS have been used to understand the genomic basis of production traits, such as feed conversion, growth rate, stress tolerance, body shape, and disease resistance, among others. Because of the polygenic nature of these traits, marker-based detection of quantitative trait loci traditionally has been used for mapping chromosomal regions associated with production traits (Gutierrez and Houston, 2017). As high-density linkage maps have been produced for various aquaculture species (see Table 3.1), genome-wide association study (GWAS) becomes the current approach for pinpointing genes determining aquacultural traits (Geng et al., 2017; Gutierrez et al., 2015).

Summing up, genomic tools have become available to fish geneticists to acquire knowledge of putative adaptive variation in Neotropical finfish. Therefore, maintaining adaptive genetic variation that contributes to a species' mechanisms for coping with environmental changes becomes a key contribution to achieving the overarching goal of conservation and protection of Neotropical fish genetic resources.

Environmental cues and expression of genes involved in reproductive physiology

Reproduction in fish is controlled by the hypothalamic-pituitary-gonadal axis, which, through the synthesis, release, and binding of hormones and neurohormones onto receptors of many cell types, modulates the individual's progress through stages of the reproductive process. Hypothalamic neurons synthesize the gonadotropin-releasing hormone, which is released into the adenohypophysis where gonadotropic cells synthesize and release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins, transported via the bloodstream, stimulate the synthesis of gonadal steroids in the follicular cells of the ovaries or the Leydig cells of the testes. Other substances and hormones—such as dopamine, melatonin, kisspeptine, gonadotropin-inhibitory hormone, and neuropeptide Y, among others—also influence the function of this axis (Levavi-Sivan et al., 2010; Zohar et al., 2010). Details of this hormonal axis are described in Chapter 14.

Studies on the biology, or more precisely, the physiological processes of a given organism, must be linked to knowledge of the environment in which the organism inhabits. For teleosts, the physicochemical characteristics of water are fundamental to understanding the physiological systems of the respective species and to predict how changes in the environment may demand adaptations to new conditions.

Animal populations can react in three ways to a local environmental change: (1) reduce the percentage of individuals that reach reproductive state, (2) migration to unaffected areas, or (3) physiological and behavioral adaptation of individuals to maintain homeostasis in the new condition (Donnelly, 1998; Helmuth et al., 2005). Allostatic capacity varies through time, both in individual and developmental dimensions. Environmental alterations may exceed the allostatic capacity of animals and cause stress, which is the set of reactions of the organism in response to disruption of homeostasis. This disturbance of homeostasis entails the activation of set-point adjustment mechanisms so that animals can continue to live in altered environments, and these set-point adjustments often include modulations of the expression of genes considered important to a given function, now impacted by environmental conditions (Mrosovsky, 1990).

Regarding tropical, freshwater teleosts, the effects of changes in water characteristics are studied with regard to endpoints such as growth (Lopes et al., 2001; Beux and Zaniboni-Filho, 2007; Souza et al., 2016), sexual differentiation (Nakamura et al., 1998; Blázquez and Somoza, 2010), and reproduction (Gomes et al., 2010, 2015; Honji et al., 2009; Correia et al., 2010; Kida et al., 2016; Tolussi et al., 2018a). In addition to changes in physical and chemical characteristics of the water, other actions of anthropogenic origin also may alter environmental conditions. A serious ecological problem is the fragmentation of aquatic habitats by damming of rivers for hydropower operations. Potamodromous fish, that is, species that live exclusively in rivers (Lucas and Baras, 2001), migrate upstream in the reproductive period (Agostinho et al., 2003), and can travel up to 43 km per day (Godoy, 1975). From the functional point of view, the selection of the appropriate moment for migration implies that environmental stimuli are correctly identified and "interpreted." Because the stimuli and the ability to migrate developed during coevolution of the species and its environment, then the species' current and future adaptations also depend on how the environment changes as a result of natural or anthropogenic causes (Lucas and Baras, 2001). Among the stimuli modulating migration of potamodromous species in the tropics is rainfall, which, being seasonal, is directly related to the spawning period of lotic fish (Godinho et al., 2010), which usually spawn only during short periods of the year.

Even today, there is a lack of knowledge about the swimming capacity of most freshwater fish, especially Neotropical species. Building knowledge in that regard is increasingly needed for better understanding of Neotropical fish migratory capacities and for assessing how anthropogenic changes affect the ecophysiology of these animals. Kinematic cameras and breathers are of great utility for measuring speed and swimming ability. In addition, telemetry techniques offer great

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potential for determining the real costs of migration and the functionality of fish passages (Godinho et al., 2007; Hahn et al., 2007). Nonetheless, more direct, simple, and quick analyses, for example, determination of fish muscle mass, red muscle ratio, and energy reserves, are relevant because they can give insight into speed and endurance by comparison with well-characterized models (Milligan and Girard, 1993; Moreira et al., 2002).

The interruption of spawning migration by dams can cause reproductive failure (Agostinho et al., 2007; Honji et al., 2009, 2018; Moreira et al., 2015; Branco et al., 2017; Tolussi et al., 2018b) and local extinction of species, although river tributaries sometimes serve as alternative sites for reproductive migration and spawning if precipitation, water levels, and habitat are appropriate (Santos and Formagio, 2000). The effects upon individual species may affect fish communities because habitat fragmentation often has a marked effect on larger migratory species with high longevity and low reproductive potential, but in contrast may benefit small, sedentary species with short lifespans and high reproductive potential (Agostinho et al., 1999). Therefore, changes in water quality and regional anthropogenic actions can affect the structure of ichthyofaunal communities, with loss of species richness (Agostinho et al., 1999; Godinho et al., 2007). For example, this problem has been detrimental to the ichthyofauna of the Upper Tietê River basin, which includes the source of one of the most important rivers in the city and state of São Paulo in Brazil (Torloni et al., 1993). These phenomena also were observed in several other watersheds in Brazil; the southeastern region of the Paraíba do Sul basin is also worthy of note. Considering the magnitude of the problem, relatively few efforts have been devoted to understanding the physiological mechanisms whereby the interruption of migration (also known locally as "piracema") impairs the reproduction of fish, as studies show that interrupted migration impairs gonadal development, maturation of gametes, and spawning (Rocha and Rocha, 2006; Moreira et al., 2015).

More recently, as a result of the availability of molecular techniques that allow the investigation of more specific mechanisms underlying the physiological control of reproduction, studies on reproductive physiology have begun to diversify. The use of molecular biology techniques in gene expression studies has become increasingly frequent in animal production research (Gabriel, 2001). Use of western, northern, and southern blot assays, in situ hybridization, quantitative PCR, and DNA microarrays (Goetz and Mackenzie, 2008) are useful for advancing understanding of the physiology of different fish species. Many studies have been carried out on the expression of genes related to the control of fish reproduction (Ravaglia et al., 1997; Somoza et al., 2002; Mateos et al., 2003; Saito et al., 2003; Vong et al., 2003; Dheda et al., 2004; Kim et al., 2005; Olsvik et al., 2005; So et al., 2005; Cerdà et al., 2008; Guzmán et al., 2009; Corchuelo et al., 2017; Assis et al., 2018). Regarding freshwater Neotropical fish, most information has become available only recently (Adolfi et al., 2015; Moreira et al., 2015; Jesus et al., 2017a). Molecular genetic tools have proven useful for evaluating the effects of environmental variables on the reproductive physiology of tropical teleosts (Tolussi et al., 2018a).

Several studies have applied molecular techniques to investigate adjustments in the reproductive physiology of fish restrained from migrating. These have focused on the characid tabarana, *Salminus hilarii*, with an emphasis on the morphological and endocrine pathways involved in physiological adjustments (Honji et al., 2009, 2011, 2013; Araújo et al., 2012; Moreira et al., 2015). A comparative study of adult female tabarana in confinement and in the wild showed that the β-subunits of FSH and LH are expressed differently through the reproductive cycle (Moreira et al., 2015), thus finally explaining physiologically the success of the pituitary extract spawning method used for the past century (von Ihering and Azevedo, 1936), including successful use with tropical species (Leonardo et al., 2004, Bombardelli et al., 2006; Zaniboni-Filho and Weingartner, 2007; Caneppele et al., 2009; Caneppele et al., 2015; Nogueira et al., 2012; Sanches et al., 2016; Damasceno et al., 2017; Pereira et al., 2018) such as *S. hilari* (Honji et al., 2011). Honji et al. (2009) showed that blockage of spawning migration interrupts the final maturation of gametes and ovulation in *S. hilarii*. Analyzing the expression of pituitary gonadotropin genes, the authors reported that for reaching advanced stages of ovarian development when ovulation becomes possible, captive females exhibited lower estradiol levels than wild females, parallel to a reduction in expression of the FSH gene. This hormonal profile interfered with the negative feedback loop of estrogen in the expression of LH, thereby altering the profile of progestogen synthesis (Moreira et al., 2015).

Use of genetic tools in ecotoxicological studies with neotropical fish

Aquatic ecotoxicology studies interactions between chemical compounds in the environment and biocenosis, the association of different organisms within a closely integrated community, focusing of adverse effects at different levels of biological organization. The use of genetic tools, especially those focusing on gene expression, might prove important to the further development of aquatic ecotoxicology. This research area requires multidisciplinary efforts examining physical-chemical, molecular, toxicological, physiological, and ecological processes (Fent, 2001; Lawrence and Hemingway, 2003; Kumar and Denslow, 2017; Martyniuk, 2018).

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Endocrine-disrupting compounds (EDCs) are important drivers of physiological dysfunctions caused by different pollutants. Biomarkers often are used to detect these effects. Biomarkers are internal indicators of changes in organisms at the cellular or molecular levels, and have great potential for advancing understanding of the effects of changes in the environment and for optimizing risk-assessment processes (Bennett and Waters, 2000; Hook et al., 2014).

Molecular biomarkers have been investigated in tropical teleosts with the aim of detecting the action of pollutants upon the endocrine-driven physiology of animals, mainly as surrogates or competitors of hormones. Reportedly, estradiol causes deleterious effects in the livers of male silver catfish ("jundiá"), *Rhamdia quelen*. Moura Costa et al. (2010) suggested that the expression of vitellogenin (VTG), the activities of enzymes, such as catalase and superoxide dismutase, and the concentration of metallothionein are important biomarkers for the effects of pollutants with estrogenic action. In jundiá, exposure to sublethal concentrations of herbicides (methyl-parathion, atrazine, simazine, and glyphosate) ordinarily used in fish farming operations in southeast Brazil had a deleterious effect upon the response to cortisol, and hence are considered acute stressors (Cericato et al., 2008). Species of the characid genus *Astyanax* have been studied as model systems for the ecotoxicology of EDCs. In *Astyanax fasciatus*, plasma estradiol levels, together with the detection of VTG gene expression, are considered good biomarkers for detecting the presence of EDCs in water (Tolussi et al., 2018a). A study of ovarian proteins of *A. fasciatus* provided evidence of the presence of EDCs in wild populations inhabiting a reservoir affected by agricultural residues (Prado et al., 2011), and indicated that the insecticide Thiodan may act as an EDC on secondary ovarian follicles (Marcon et al., 2017). Weber et al. (2017) described overripening and yolk-deficient oocytes as biomarkers of estrogenic EDCs for *Astyanax rivularis*.

Molecular biomarkers also have been used in tropical species to assess the effects of insecticides. Biomarkers of oxidative stress of "matrinxã" *Brycon cephalus* were investigated and detected after exposure to organophosphate insecticides by Monteiro et al. (2006). The "sábalo" or "curimbatá," *Prochilodus lineatus*, has been used as a model species for investigating the effects of insecticides (Vieira et al., 2014, 2016, 2018) and herbicides (Pereira et al., 2013; Moreno et al., 2014; Navarro and Martinez, 2014) at the molecular level. The South American cichlid *Cichlasoma dimerus* also has been an important model for studying the effects of insecticides (Da Cuña et al., 2016) and organic compounds (Rey-Vázquez et al., 2009; Genovese et al., 2011, 2012, 2014) at the molecular level.

The use of molecular biomarkers also has been used to detect the effects of metals upon freshwater teleosts. Exposure to tannery effluents, which are rich in chromium (Lunardelli et al., 2018), copper (Nascimento et al., 2012; Simonato et al., 2016), aluminum (Galindo et al., 2010), lead (Monteiro et al., 2011; Ribeiro et al., 2014), cadmium (Silva and Martinez, 2014), nickel (Palermo et al., 2015), and combinations of metals such as zinc, manganese, and iron (Oliveira et al., 2018), triggered alterations in oxidative stress enzymes in *P. lineatus*. In tambaqui, *C. macropomum*, subchronic exposure to manganese triggered different responses in oxidative stress enzymes and lipo-peroxidation effects, depending upon the tissue analyzed (Gabriel et al., 2013). Studies on the activity of transport enzymes, hypoxia tolerance, and other metabolic biomarkers of tambaqui showed that physiological impacts of the ingestion of copper in the diet were minimal, but that dietary cadmium increased hypoxia tolerance (Giacomin et al., 2018,). However, a combination of aluminum and manganese triggered alterations in oxidative stress enzymes in male *Astyanax altiparanae* during the reproductive season (Abdalla et al., 2019). Metals also have been considered EDCs in some teleosts. In Nile tilapia, aluminum was considered an EDC, decreasing FSH and LH gene expression (Narcizo, 2014) as well as decreasing plasma levels of 17α-hydroxyprogesterone and cortisol in females at the gonadal maturation stage (Correia et al., 2010). In turn, aluminum increased the testosterone levels of males while manganese increased estradiol levels during the reproductive season in females *A. altiparanae* (Kida et al., 2016).

The responses to stress at the cellular level in general are mediated by heat shock proteins (HSPs), which are highly conserved among most organisms (Iwama et al., 1998). Study of HSPs in *Brycon amazonicus* showed that exposure to phenol compromises their ability to exhibit physiological responses to a stressor (Hori et al., 2008). The organic compound benzo(*a*)pyrene A increased DNA damage in the liver, gills, and blood cells of *P. lineatus* (Santos et al., 2018).

These studies show that genetic tools can be widely used as valuable biomarkers to assess the sublethal effects upon fish of exposure to contaminants in water. Genetic biomarkers are reliable to detect early biological effects in the environment and can be used as a first approach in ecotoxicological studies, followed by genotoxic, structural, and populational effects (Anderson et al., 1994).

Triploidy induction in fish

The application of chemical, thermal, or hydrostatic pressure shocks to newly fertilized fish eggs can suppress ejection of the second polar body or first cell division, thereby inducing changes in the number of chromosome sets. Such chromosome set manipulations can result in individuals that are gynogenetic (having all inheritance from the mother), androgenetic

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(all inheritance from the father), or polyploid (having extra sets of chromosomes). Such procedures have been used in many fish, particularly farmed species such as salmonids (Bonnet et al., 1999; Johnston et al., 1999), carp (Basavaraju et al., 2002), and catfish (Manickam, 1991; Linhart and Flajshans, 1995). Most attention has been paid to the induction of triploidy because it enables production of sterile animals, thereby optimizing growth given that triploid fish will redirect energy reserves from the maturation of gametes (Ribeiro et al., 2012) to growth (Henken et al., 1987). In addition, triploid fish escaping from aquaculture operations would not be able to reproduce in the wild. The possession of an extra chromosome set has physiological consequences for animals, and examination of triploid individuals provides novel insights into fish physiology, a line of research best developed for salmonids. Although triploid fish are sterile, they may show varying degrees of gonadal development, and they have larger but fewer cells in most tissues and organs (Benfey, 1999), including the central nervous system and sensory organs (Small and Benfey, 1987). The aquacultural advantages of producing triploid organisms has been evaluated physiologically, and although many authors consider the physiologies of diploid and triploid animals very similar, stressors such as inadequate farming conditions or inappropriate environmental variables affect the performance of triploid animals more intensely than diploids (Maxime, 2008). Experiences with triploid salmonids in aquaculture show that they tend to have reduced survival and growth compared to diploids, and present signs of chronic stress (Benfey and Biron, 2000; Benfey, 2001).

Special attention has been given to the induction of triploidy in jundiá, a Neotropical catfish farmed in southern Brazil that presents early sexual maturation (Narahara et al., 1985). Experiments on triploidy induction in *R. quelen* have used pressure (Huergo and Zaniboni Filho, 2006), heat (Vozzi et al., 2003), or cold (Silva et al., 2007) shocks, the last yielding higher rates of triploidy. Triploidy increased the size and volume of erythrocytes but decreased the number of circulating erythrocytes, leucocytes, and thrombocytes (Fukushima et al., 2012). Lymphocytes were the most predominant white blood cells in diploid fish while monocytes predominated in triploid fish, which led the authors to recommend evaluation of resistance to infection in triploids. Weiss and Zaniboni-Filho (2009) showed that triploid juvenile jundia were more sensitive than diploid fish to ammonia in the first 48 h of exposure, although the accumulated mortality in both groups was similar after 96 h of exposure. Spontaneous triploids have been registered at low frequencies in natural populations of *R. quelen* and other members of the genus (Garcia et al., 2003; Garcia et al., 2010; Tsuda et al., 2010), most likely as the result of fertilization of eggs that did not naturally eject the second polar body.

Spontaneous triploids are known in about a dozen other Neotropical fish in a wide range of evolutionary lineages, including five of the genus *Astyanax* (Morelli et al., 1983; Fauaz et al., 1994), *Gymnotus carapo* (Fernandes-Matioli et al., 1998), *Poecilia formosa* (Lamatsch et al., 2000), and *Characidon gomesi* (Centofante et al., 2001). These studies have focused on karyology and systematics and have not addressed the degree to which natural triploids exhibit the same physiological features as diploids or induced triploids.

Transgenic fish in physiological studies

Biotechnological advances through the past three decades have provided important tools for gene-level manipulation of living organisms. Transgenesis involves the transfer of a trait from one organism to another by introduction of the gene encoding that trait into the receptor organism, enabling new, stable, and genetically defined characteristics to be incorporated into the recipient organism.

An ever-increasing number of research projects have applied gene transfer technology to fish. In addition to being commercially important, especially for farming purposes, teleost fish have certain reproductive and biological characteristics—high fecundity, large eggs, and external fertilization and embryogenesis—facilitating gene transfer procedures (Zhu and Shu, 2000). By achieving expression of the transferred gene, one or more characteristics can be modified to increase productivity and reproductive efficiency for farming purposes, yielding a true-breeding transgenic line in just a few generations (Horváth and Orbán, 1995). Wu et al. (2003) postulated that genetic manipulation of fish would be used in the future to increase commercial aquaculture production. The first government approval of commercial sale of a transgenic fish for human consumption occurred in 2015 (https://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/ucm466214.htm). The AquAdvantage salmon, produced by AquaBounty Technologies (Maynard, Massachusetts, United States), is a transgenic Atlantic salmon *Salmo salar* that expresses high levels of growth hormone (GH) and reaches commercial size in 16–18 months, half the time required for conventionally bred salmon. The fish was first sold commercially in Canada in 2017.

Gene transfer has been performed on >35 fish species, most of them important for aquaculture (Zbikowska, 2003). Although the technology has been widely applied for its potential value to aquaculture (Dunham, 2004), it also has opened a field of research using genetically modified model systems, often zebrafish, *Danio rerio*, for studying gene regulation, physiological pathways, and embryonic development (Alestrom et al., 2006).

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Because of its simplicity and reliability, the microinjection of DNA into newly inseminated fish eggs remains the most widely employed gene transfer technique (Udvadia and Linney, 2003; Zbikowska, 2003). Maclean et al. (2002) argued that in Nile tilapia, no technique has proven superior to microinjection. However, microinjection almost invariably produces mosaic individuals; that is, because of delayed integration of the gene construct into the host genome, the transgene integrates into only a portion of cells or tissues in the embryo, precluding germline transmission of the introduced gene to the offspring if there was no integration into gonadal tissues (Maclean, 1998). Therefore, delayed integration and mosaicism increase the effort required to successfully produce at least some germline-transgenic individuals that prove successful founders of transgenic lineages. To deal with the problem of mosaicism on a practical level, marker transgenes may be incorporated into or coinjected with the gene construct of interest to mark prospective founders of transgenic lineages. Marker genes such as luciferase, β-galactosidase (*lacZ*), chloramfenicol acetyl transferase (*CAT)*, neomycin phosphotransferase (*neo*), and green fluorescent protein (*GFP*) can be used to ease the identification of transgenic individuals in the first generation (Maclean, 1998). Among such markers, the *GFP* gene, isolated from the jellyfish *Aequorea victoria*, requires no exogenous substrate to analyze its activity and allows direct in vivo visualization of the marker phenotype and selection of transgenic individuals without taking a tissue sample or sacrificing the animal (Amsterdam et al., 1995).

Several gene transfer techniques were developed with the aim of increasing the efficiency of integration of transgenes into the host genome, and to produce large number of first-generation nonmosaic transgenics. To increase integration efficiency, strategies that ease transgene transport to the nucleus and insertion into the host genome have been developed, including the use of small peptides associated with transgenes termed nuclear localization signals (Collas and Alestrom, 1997). For the same purpose, adenoviral vectors (Chou et al., 2001; Hsiao et al., 2001), the enzyme meganuclease I-*Sce*I (Thermes et al., 2002), transposons (Grabher et al., 2003) and nuclear transfer (Jesuthasan and Subburaju, 2002) also have been used.

Toward the goal of producing transgenic fish at a large scale and increasing the production of nonmosaic transgenics in the first generation, some approaches have been based on manipulation of semen. Among them is electroporation, which uses an electric current to promote the entry of exogenous DNA into sperm cells. After electroporation, the transformed semen is used for in vitro fertilization (Müller et al., 1992; Lu et al., 2002). In addition, direct genetic manipulation of gonadal tissues in vivo also enables increased transgenic production (Lu et al., 2002).

Gene transfer techniques have been supplemented by a more precise and powerful set of techniques collectively termed gene editing. This approach is advantageous because the vectors can target a transgene to integrate into a specific site in the host genome and also because of a higher transformation success rate. The first gene-editing experiments in fish were performed with zebrafish, starting with targeted mutagenesis using zinc finger nucleases (ZFN; Meng et al., 2008; Doyon et al., 2008), followed by the more efficient transcription activator-like effectors (TALENS; Huang et al., 2011). Dong et al. (2011) used ZFN technology to examine muscle growth mechanisms in yellow catfish, *Pelteobagrus fluvidraco*, and Yano et al. (2013) to induce targeted mutagenesis of the *sdy* gene of rainbow trout, *Oncorhyncus mykiss*, to confirm its function in sex determination. The clustered regularly interspaced palindromic repeats/Cas9 (CRISPR-Cas9) system, now most frequently used for gene editing (Wiedenheft et al., 2012), originally was identified in bacteria and archaea, which utilize this system for protection against bacteriophages and foreign DNA (Horváth and Barrangou, 2010). The system has been applied for gene editing in a wide variety of plants and animals, including zebrafish and tilapia (Hwang et al., 2013; Li et al., 2015). This technology is so efficient in zebrafish that it can induce gene-specific, biallelic mutations in the first F_0 generation (Jao et al., 2013). Edvardsen et al. (2014) used the CRISPR-Cas9 system to produce complete gene knockout Atlantic salmon in the F_0 generation. Wargelius et al. (2016) subsequently knocked out the *dnd* gene to ablate germ cells of Atlantic salmon, with the ultimate aim of producing reproductively confined aquaculture fish.

Regardless of the technique, a wide variety of genes have been used for producing transgenic fish with the aim of influencing physiologically important traits for aquaculture, such as growth (reviewed by Hallerman et al., 2007), gonadal maturation (Uzbekova et al., 2000; Wargelius et al., 2016), resistance to freezing (Hew et al., 1999; Fletcher et al., 1992a,b, 2004), and disease resistance (Zhong et al., 2002; Dunham et al., 2002; Mao et al., 2004). These studies pioneered a novel research field with considerable potential for genetic improvement for farming purposes.

Growth rate has been the most frequent target for genetic manipulation. Early studies with GH gene transfer (Maclean and Talwar, 1984; Zhu et al., 1985) showed growth enhancement, with as much as 11-fold enhancement for coho salmon, *Oncorhynchus kisutch* (Devlin et al., 1994), and 35-fold for mud loach, *Misgurnus mizolepis* (Nam et al. 2001). The core of studies on GH transgenesis led to insights into the physiology of growth and its linkages to other physiological processes in the host. The pathway through which the growth process becomes manifested begins with the synthesis of GH in the pituitary gland, which is released into the bloodstream where it binds to growth hormone receptors (GHR) on the membrane of target cells, activating genes involved in the development of biological responses to GH. Among these, the most important are those encoding insulin-like growth factors—IGFs (Schindler and Darnell, 1995; Ihle, 1996). IGFs are small

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polypeptides produced mainly in the liver that bind to receptors on a wide range of cells in the host, exerting a direct influence on animal growth and development (Yakar et al., 1999). The biological responses of GH are set up and controlled mostly by the so-called somatotropic axis, in which IGF-I is the main mediator of the physiological action of GH (Moriyama et al., 2000, Butler and Le Roith, 2001).

Although manipulation of the GH gene shows promise regarding growth in fish, its excess may lead to unintended side effects, termed negative pleiotropies. Reviewing the effects of GH transgenes on farmed fish, Devlin et al. (2006) and Hallerman et al. (2007) noted that elevation of bodily GH content increased rates of protein synthesis and lipid mobilization, affecting not only growth but also feed conversion efficiency, metabolic rate, body composition, head and body morphometrics, osmoregulation, immune response, and age at maturity, with other modifications particular to species and transgenic lines. Because of heightened feeding motivation, transgenic fish often proved more active, aggressive, and willing to risk exposure to predation. The swimming ability of some transgenic lines was reduced. While limited in scope, studies suggested that fish of some transgenic lines show restrained reproductive behavior. As a result of GH overexpression or administration, a biologically meaningful increase in metabolic rate and oxygen consumption was reported in Atlantic salmon (Cook et al., 2000; Herbert et al., 2001) and Nile tilapia (McKenzie et al., 2000, 2003). Mori et al. (2007) registered changes in the expression of immunological, reproductive, and growth-related liver genes in masu salmon, *Oncorhynchus masou*. Cnaani et al. (2013) determined the responses of juvenile GH-transgenic and triploid Atlantic salmon to one week of fasting or low dissolved oxygen (1.5–2.0 ppm). After fasting, transgenic fish had higher levels of sodium and chloride than other genotypes, suggesting osmoregulatory difficulties. Immediately after anoxic challenge, transgenic fish exhibited higher hematocrit, pCO_2, glucose, and sodium levels than other genotypes. Wild-type fish maintained homeostasis more effectively than transgenic or triploid fish, exhibiting smaller changes in all measured stress-response parameters.

Not long ago, only mammalian models were available for GH studies on physiological processes. However, a transgenic fish model—zebrafish that overexpresses GH—has been developed, the first lineage of transgenic fish produced in Brazil (Figueiredo et al., 2007a). This lineage, called F0104, besides overexpressing the *GH* gene, also carries the *GFP* marker gene, which greatly facilitates the identification of transgenic individuals (Fig. 3.3). Fish from the F0104 lineage have been studied not only regarding growth and regulation of the somatotrophic axis, but also concerning different physiological aspects directly influenced by GH, such as metabolic acceleration, production of active oxygen species, oxidative stress, aging, and muscle tissue structure. Studies with different genotypes from the F0104 lineage have shown a significant increase of growth rate in hemizygous individuals (Fig. 3.4) and also in the expression level of the GHR and insulin-like growth factor I (IGF-I) in the liver. However, the growth of homozygotes that express twice the GH as hemizygotes does not differ from nontransgenic controls, suggesting an optimal hormone level under controlled feeding conditions (Figueiredo et al., 2007b). These homozygotes also exhibited a catabolic state, probably due to the effects of excess circulating hormone.

Studzinski et al. (2009) reported that the lack of growth proportional to the GH level in homozygous individuals of the F0104 lineage may be related to the energy cost of activating a negative regulatory mechanism of the somatotrophic axis based on increased hepatic expression of GH intracellular signaling blocking proteins, the SOCS (suppressor of cytokine signaling). Alternatively, overexpression of the GH protein may have driven growth to its full physiological potential, as also inferred for GH-transgenic rainbow trout (Devlin et al., 2001).

As reported by Rosa et al. (2008), homozygous individuals of the F0104 lineage present increased generation of reactive oxygen species, probably related to the anabolic effect of GH and as a result of increased oxygen consumption and metabolic rate. This overexpression caused a negative regulation of the expression of genes from the antioxidant defense system in muscular and hepatic tissues. Using this same transgenic lineage, Rosa et al. (2010) demonstrated a correlation between the decreasing expression of genes from the antioxidant defense system and an aging phenotype associated with

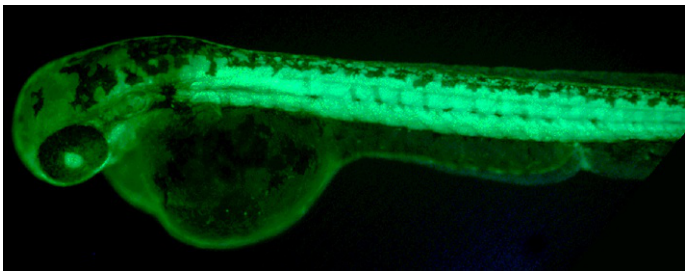

FIG. 3.3 A transgenic zebrafish (*Danio rerio*) larva from the F0104 lineage expressing both GH (growth hormone) and GFP (green fluorescent protein) under control of the β-actin promoter. *(Photo: Luis Fernando Marins).*

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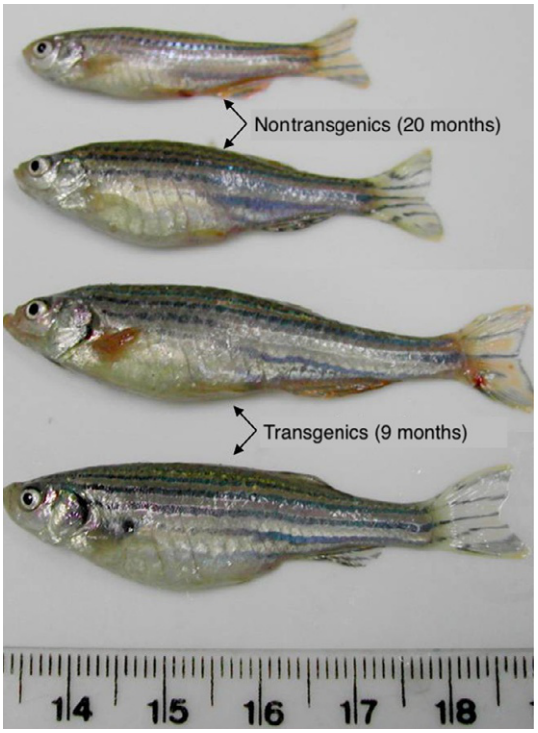

FIG. 3.4 Growth comparison between GH-transgenic and nontransgenic zebrafish (*Danio rerio*).

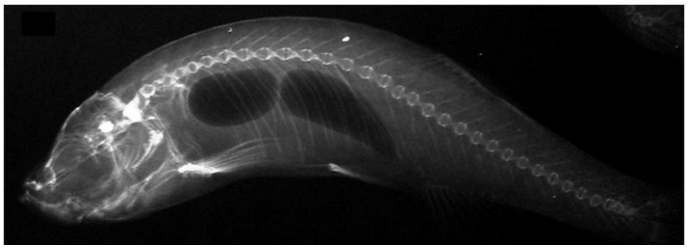

FIG. 3.5 Effect of GH overexpression on spinal curvature of transgenic zebrafish (*Danio rerio*) (Rosa et al., 2010).

spinal curvature. This effect is directly linked to the pathological process known as sarcopenia, related to reduced muscle regeneration capacity, thereby indicating a clear relationship between GH overexpression and aging (Fig. 3.5).

The muscle structure of the transgenic F0104 lineage was studied by Kuradomi et al. (2011). Histological analyses unveiled that transgenics presented marked muscular hypertrophy compared to nontransgenics, with a significant increase in the percentage of thick muscle fibers (Fig. 3.6). The transgenic hypertrophy was independent of muscle IGF-I induction, probably as a direct effect of excess circulating GH and/or IGF-I.

Because GH is related to salinity tolerance in fish, Almeida et al. (2013) tested whether the osmoregulatory capacity of zebrafish, a freshwater species, would be modified by GH-transgenesis. They transferred GH-transgenic zebrafish from freshwater to brackish water, 11 ppt NaCl. Higher mortality was recorded in the transgenic population than in nontransgenic fish. Genes encoding Na^+,K^+-ATPase, H^+-ATPase, plasma carbonic anhydrase, and cytosolic carbonic anhydrase were upregulated in the gills of transgenics kept in freshwater. The GHR gene was downregulated in the gills and liver of both transgenic and nontransgenic fish exposed to 11 ppt NaCl, and IGF-I expression was downregulated in the liver of nontransgenics and the gills of transgenics exposed to 11 ppt NaCl. In transgenics, all osmoregulation-related genes and the citrate synthase gene were downregulated in gills of fish kept in brackish water while lactate dehydrogenase expression was upregulated in the liver. Na^+,K^+-ATPase activity was higher in gills of transgenics exposed to 11 ppt NaCl as well as the whole-body content of Na^+. These findings collectively support the hypothesis that GH-transgenesis increases Na^+ import capacity and energetic demand, promoting an unfavorable osmotic and energetic physiological status and making this transgenic fish intolerant of hyperosmotic environments.

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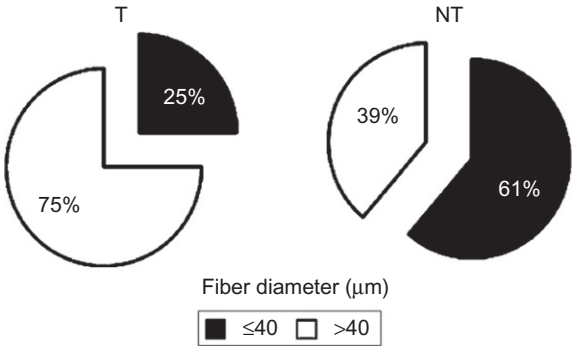

FIG. 3.6 Proportion of thin (≤40 μm) and thick (>40 μm) muscle fibers in transgenic (T) and nontransgenic (NT) zebrafish, *Danio rerio* (Kuradomi, 2009).

Because GH is an important regulator of immune functions in vertebrates, Batista et al. (2014) examined the effects of the overexpression of GH on immune function. GH-transgenic zebrafish exhibited 100% mortality when immunosuppressed with dexamethasone, revealing a weakening of the immune system. The thymus and head kidney, important to immune function in fish, were reduced in size in transgenic zebrafish. The phenotypic expression of CD3 and CD4 thymocytes was reduced in transgenic zebrafish. Decreases were recorded for the expression of the *RAG-1* (60%), *IKAROS* (50%), *IL-1b* (55%), *CD4* (60%), and *CD247* (40%) genes, indicating that the development of innate and acquired immunity functions was harmed. Hence, excess GH impaired immune functions in GH-transgenic zebrafish.

GH transgenesis results in increased growth and food intake and, consequently, increased metabolic rates in fish. Dalmolin et al. (2015) examined the effect of GH overexpression on appetite control mechanisms in fasting and fed GH-transgenic zebrafish. The feed ingestion of transgenic individuals was significantly higher and faster than that of nontransgenic siblings. Gastrointestinal (GT) cholecystokinin contributed substantially to communication between the peripheral and central control of food intake, as unveiled by gene expression analysis. Analysis of the expression of brain genes revealed that transgenic animals had downregulation of two strong and opposite peptides related to food intake: the anorexigenic proopiomelanocortin (pomc) and the orexigenic neuropeptide Y (npy). The downregulation of *pomc* in transgenics, as compared to nontransgenics, is an expected trend as the decrease in an anorexigenic factor would keep the transgenic fish hungry. GH elevates blood glucose, and NPY responds to humoral factors such as glucose, leading to downregulation.

All these findings indicate a series of physiological side effects caused by excess GH, which may have important implications for the application of this technology in aquaculture. These observations point to the need for a thorough characterization of transgenic lines before aquacultural application of transgenesis, in a way that increased growth may be obtained without negative pleiotropies on the fish (Hallerman et al., 2007). Optimal, rather than maximal, levels of GH expression may be critical to achieving growth rate enhancement without unwanted pleiotropic effects. Other approaches might also be envisioned.

An alternative to increasing levels of circulating GH to achieve growth rate enhancement might be the application of transgenesis to raise the levels of GHR. This approach would introduce two interesting possibilities. First, the animal would be able to regulate hormone levels according to its momentary needs, allowing the optimal use of available energy. This is important because in the case of the transgenic GH models currently used, excess circulating GH cannot be regulated by the organism, which implies growth without the usual homeostatic controls, even under unfavorable conditions, leading to undesired metabolic effects. Second, the use of tissue-specific promoters (i.e., gene regulatory sequences) might direct the effect of circulating GH to tissues of greater interest for increasing the productivity of the cultured organism, for example by increasing the expression of GHR in skeletal muscle tissue. A transgenic fish with such characteristics could, in theory, regulate its circulating GH levels to maintain homeostasis without negative pleiotropies and, at the same time, maximize the use of naturally occurring circulating hormone in the target tissue through an increase in the amount of receptors in the cell membranes. Should this model prove true, the expected result would be fish with more muscle tissue, but with normal levels of circulating GH. This hypothesis was tested by Figueiredo et al. (2012), who produced a transgenic zebrafish line overexpressing GHR in skeletal muscle. Even with the transgenic zebrafish expressing 100 times more GHR than nontransgenics and exhibiting hyperplasia, the fish had no supranormal weight gain. The authors concluded that GHR overexpression did not induce muscle growth because of impairment of the GHR/IGF-I axis by suppressors of cytokine signaling (SOCS proteins). It thus seems that hypertrophy and hyperplasia are expressed through two different pathways, both triggered by GHR activation but regulated by different mechanisms.

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Taking into account that the overexpression of GHR leads to the expression of somatotropic blocking proteins, another alternative would be to manipulate the GHR molecule to make it constitutively activated; that is, the somatotropic axis could be activated independently of circulating GH and its side effects. Ahmed et al. (2011) demonstrated that it is possible to produce a constitutively activated GHR (CA-GHR) by replacing the extracellular portion of the receptor (the DEC and DJ domains) by a leucine zipper derived from a transcription factor known as c-jun. The authors produced a transgenic zebrafish line overexpressing the CA-GHR and observed higher growth of transgenics than nontransgenics. This study introduces the possibility of improving growth performance through a hormone-independent mechanism. The design of new molecules related to growth signaling may become an interesting approach to the development of new lineages for aquaculture.

To summarize, transgenic fish models have contributed significantly to the advancement of knowledge of key physiological mechanisms of vertebrates. This advance in knowledge, associated with the development of modern techniques of manipulation and editing of genes, has opened a new avenue of opportunity for new technological developments that can be applied to aquaculture. Thus, transgenic fish models can contribute to the advancement of the basic physiological sciences while supporting new technologies for animal protein production.

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Chapter 4

Behavior and welfare

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Introduction

In this chapter, we focus on the issue of fish welfare, starting by presenting the basic structure of fish behavior to better understand the nature of fish, a necessary goal for evaluating considerations about fish welfare. The examples presented are focused on Neotropical fish, but they can be extrapolated to cover similar issues for other species, too.

Most studies of fish behavior have been limited to describing what these animals do, mainly focusing on the motor component of the behavior. Although this approach is valuable, it is certainly not enough. The study of behavior deserves understanding principles and laws that explain what fish do and why they do it; it involves the study of motor patterns, motivations, and evolutionary, genetic, and environmental influences. As a natural unfolding, understanding animal behavior leads to concerns about the welfare of these organisms. Although this is a current subject, a brief visit to its historical roots reveals the depth and antiquity of the theme, but also better allows us to understand the current scenario. To support the ideas that the science of well-being can bring to man-fish interactions, this theme was developed as a complement to the understanding of behavior. The more usual approaches were visited in an attempt to construct a structured and coherent theoretical framework that would give meaning to human actions in relation to fish—a framework that is also perfectly valid for many other animals.

More than just a didactic text on the study of welfare, we present here a critical-reflective view on the main ideas of this branch of science. This analysis reveals profound implications on how to treat fish. The natural unfolding rests on the questions of what is necessary to consider fish welfare and how that relates to the ethics of humans' treatment, protections, and use of such animals.

Basis for the study of behavior

In this section, the bases for the behavioral study were analyzed, answering such questions as "What is behavior?," "How is it controlled?," and "What is the behavioral structure?," and discussing some examples of the motor, psychological, and physiological interactions in the behavioral issue.

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What is behavior?

One definition of behavior would be that it is what humans can perceive of an animal's actions (in terms of motor movements). However, while aborting motor reaction—that is, opting for something that looks more like inaction—a fish might be expressing the right response (e.g., being quiet while a predator is nearby). It might express intense systemic responses such as a freeze (freezing), a complete cessation of movement (except for gills and eyes) by the fish while at the bottom of the tank, as a result of high stress, anxiety, or part of a submissive behavior (Kalueff et al., 2013). To contemplate a more comprehensive view of behavior, following the concepts of neurophysiologists Miguel Rolando Covian and César Timo-Iaria, behavior can be defined as an object of physiology due to its role in mediating the brain-environment relationship. Their explanation relies on the assumption that behavior, including that of the mind, is supported by neuroendocrine controls. This expands a more classical consideration of behavior in terms of motor responses because it includes the physiological and biochemical processes underlying motor reactions and conceives of these processes as behavior in itself.

Accordingly, behavior comprises two types: motor and neurovegetative (in which hormonal controls were included). Although the motor component has been widely considered in behavioral studies, the other components have been relegated usually to physiology and biochemical studies, or even to psychology. Such compartmentalized views are not reasonable approaches, particularly given the current knowledge and circumstances. The motor component is expressed by skeletal muscle activities (swimming, staying still, turning around, biting, etc.). The underlying machinery to support such motor patterns is referred to as support adjustments (and the hormonal and neurohormonal controls were included). The other underlying machineries support specific adjustments specific to the motor behavior expressed (for instance, feeding, running, playing, copulating, etc.) and are called characteristic components of the behavior.

For example, in the reproductive behavior of Nile tilapia (*Oreochromis niloticus*), the male establishes a territory, builds a nest, seeks a female, and exhibits a ritual until he is accepted by the female, after which spawning may occur. The spawning is characterized by the elimination of the female gametes in the nest, followed by the elimination of the male gametes, which generally guarantees fertilization. The female then takes the eggs into her mouth and initiates the oral incubation until the eggs hatch, following the development process of the larvae. In this case, the male's motor patterns—defending territory, building a nest, and attracting the female—are part of the motor component of the behavior. Parallel to this, internal adjustments took place that maturated gametes and put them in conditions to be eliminated at the right moment. This occurs with the elimination of gametes, the product of characteristic neurovegetative adjustments. For all this to happen, it is necessary for the control systems, especially the neural, to be properly prepared with oxygen and glucose support. Note that the support setting is, in general terms, the same for the other behaviors, varying only in their location in the nervous and muscular systems. The characteristic component, however, varies specifically as a function of the behavior. In the case of the reproductive behavior described above, the physiological and biochemical adjustments specifically related to the maturation of the reproductive cells and their release on the nest are the characteristic components of the reproductive behavior (without them, the complete reproductive behavior does not occur or does not work properly). This more interdisciplinary and holistic view of the behavioral processes better fits our understanding of what animals do and why they do it.

The traditional division of the organism into subsystems is inherited from traditional education. It emphasizes that pharmacology is not physiology, that both are not biochemistry, and that nutrition and digestive processes are different chapters. While all this may be true on a certain level, the division is good only for understanding the parts but fails to help understand the whole. Thus, to understand and properly interpret animal behavior, it is critical to consider the organism as a whole; this organism is inside its environment and is strongly marked by its natural selection throughout the evolutionary process. Moreover, psychological processes are also an inherent part in the whole organisms and should not be treated separately. Distortions in this approach can lead to misinterpretations, which may result in misconduct relating to the organism. In the case of fish farming, how is it possible to produce a fish properly without knowing much of its life? Remember that the more you know about a system, the more you can interact with it.

Behavior structure

To better understand fish behavior, it is necessary to introduce at least the most common types of behavior. This helps the understanding of what animals are doing, which is crucial for understanding why they are doing it.

Reflex behavior

Reflex behaviors are involuntary, without necessary action of the encephalon. A reflex system is composed of five elements: (a) receptor (which transduces the stimulus to an action potential); (b) sensory (afferent) nerve; (c) synapse; (d)

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motor nerve (efferent); and (e) target organ (e.g., skeletal muscle, smooth muscle, or even a gland). Fish, when swimming, usually exhibit a curling behavior with their bodies; this is reflexively controlled but can be modified at any moment by voluntary action by the higher centers (stopping or intensifying, according to the stimulation received by the brain). Genetic control of such responses is evident because these neural pathways are genetically built, in the formation of the individual. A reflex arc conducts the stimulus and brings the response, which is usually appropriate to the context because this kind of response is strongly inflexible but shaped through evolutionary ways.

Taxis

Taxis is also an involuntary reactive behavior in response to a characteristic stimulus. It is a response to environmental stimuli and is associated with the stored memories of the fish, as for example in the anadromic migrations. The main types described in fish are: (a) *phototaxis*, response to a light source (e.g., some fish larvae approach the light while others move away from it. This response may even change with development, such as in Atlantic salmon larvae, which begin as photonegative (flee from light) and subsequently become photopositive (Ceccarelli et al., 2000; Turner and Huntingford, 1986)); (b) *reotaxis*, a response to the flow of water, positive when in the same direction and negative in the opposite direction (e.g., in anadromous migration, the reotaxis is negative (upstream), and in the catadromous migration it is positive (downstream)); (c) *thigmotaxis*, a response to touch (contact) with elements of the environment (e.g., the animal seeks to remain in the corner of the aquarium (contact with the surfaces of this environment) or lean against a stone or other objects); (d) *chemotaxis*, a response to certain chemical agents (e.g., common in the attraction that some larvae have to the smell of the parents); and (e) *geotaxis*, response to gravity (e.g., larvae may have negative geotaxis, tending to be in the vicinity of the surface of the water, which can provide them with certain feeding conditions).

Inborn behavior (instinctive)

Inborn behavior is associated with genetic influence (heritability). It is already appropriate the first time it is displayed; there is no need for training or other previous experience. In contrast to the reflex, with inborn behavior differences in presentation time between the triggering stimuli may cause changes in the intensity of the behavior. This is a fundamental difference that places these behavioral patterns as a different category of behavior. These behaviors are called *fixed patterns of action*, as they result in a standardized response when stimulated by specific stimuli, called *signal stimuli*. Among fish, there are several fixed patterns of action, including the mating ritual, nest building, care for offspring, and antipredatory responses, among others. They are more inflexible because of genetic determination but might be slightly influenced by experiences and maturation.

Learning

Learned behaviors are the results of previous experiences. It is considered *learning* when, after some experience, behavior changes but cannot be attributed to changes in motivation (e.g., reduced hunger or thirst), maturation (e.g., hormonal changes), injury, or development (age). It follows then that learning involves the participation of memory processes. For example, in habituation, when a fish in laboratory conditions perceives the presence of people, it changes its behavior accordingly (e.g., they stop swimming and stay close to the substrate). However, as this sequence is repeated with no disadvantage to the fish, they gradually get "used to" people and do not react as they did previously. Such changes in behavior are mediated by conditioning.

Classical or Pavlovian conditioning

Classic conditioning was discovered by Ivan Petrovich Pavlov, and is thus sometimes called *Pavlovian conditioning*. Conditioning is the process that creates an association between stimuli and responses. From conditioning, one stimulus comes to mean what another meant, through simple pairing. Consider that an animal exhibits naturally a certain response to a stimulus (e.g., a hungry fish swims to a food released in an aquaculture tank). If, immediately before feeding, the fish feeder introduces the sound of a horn into the water, after some repetitions of this feeding-sound pairing, the fish will swim to the feeding place immediately after hearing the sound before the food is released. Such a transference of information (from the food to the sound) by pairing both stimuli is the conditioning that enables learning.

Although learning by Pavlovian conditioning had been widely known in fish and other vertebrates, the conditioning of a hormonal response in fish was first specifically demonstrated in the Nile tilapia (*O. niloticus*). In the study group, confinement stress, which increases plasma cortisol, was provided once a day during 9 consecutive days paired with 1 minute of light (four other groups controlled for undesired factors). On the 10th day, light emission without confinement was sufficient to provoke a plasma cortisol rise (Moreira and Volpato, 2004). Later that same year, this hormonal conditioning was

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also demonstrated in the rainbow trout by Moreira et al. (2004), who added a second experimental phase providing extinction of the conditioned response in 2–3 weeks. This demonstration of memory-induced stress was suggested to understand and redefine stressors in aquatic organisms, including a category in which the stressor is not a real agent but only an evoked reminder.

Operant conditioning

Operant conditioning was discovered by Burrhus Frederic Skinner. In this case, learning occurs because of an association between an animal's behavior and the immediate consequences for that animal. For instance, consider that reinforcement drives the frequency of a particular behavior. Thus, a stimulus that increases the frequency of a behavior is considered a positive reinforcement for that behavior while the negative reinforcements reduce the frequency of the behavior. For this conditioning, reinforcement must occur soon after the behavior has been emitted. Under such conditions, the animal will associate its behavior with the consequences; thus, operant learning enables modulation of behavior by reinforcements (positive or negative).

To condition a fish in this way, it is necessary that it receive a positive reinforcement several times until this leads to the desired final behavior. For example, if the intention is to condition a fish to push a lever for food, there is a need for food to be a positive reinforcement. However, the food will only be a positive reinforcement if the fish is hungry. Thus, the offer of food should be in small amounts, so that the conditioning can be achieved before the fish is satiated. The procedure is simple, although it can be somewhat laborious, requiring patience and caution. Initially, give a few pellets of food to the hungry fish repeatedly, with short time gaps between them. Now, stop the feeding and note its behavior. It will naturally begin to move in search of the food that stopped appearing "for no reason." When the fish approaches the lever region, immediately release more food. After some repetitions of this conditioning, the fish will learn that the food will appear when it is close to the lever. At this point, stop feeding and observe the behavior of the fish. When it eventually heads close to (or even touches) the lever, immediately give it more pellets. Gradually, the fish will learn that food is associated with its approaching or touching the lever. Do this repeatedly until the fish learns that touching the lever causes food to be released. In addition, this process can also occur casually. By leaving a lever in the fish tank, fish may, out of curiosity or chance, touch the lever, which will automatically release food. If this happens multiple times, the fish will soon learn that the action of touching the lever releases the food. If the fish are hungry, they will then begin to touch the lever intentionally to get the food. They will have learned by operant conditioning.

In the same way, it is possible to model the behavior of the fish by means of negative reinforcement. Each time the animal does something that is not wanted, a negative reinforcement is offered (e.g., a sound, an intense light). Gradually, the fish ceases to perform this behavior to forestall the negative reinforcement. Both positive and negative reinforcements are ways to condition fish and other animals as well as human beings.

Aggression and territoriality

Group life has many advantages for animals, such as reduced risk of predation (Bertram, 1980; Pulliam, 1973), increased ability to find food (Ward and Zahavi, 1973; Wrangham, 1980), and greater opportunities to find reproductive partners (Lee, 1994). However, many problems must be resolved to sustain a stable group and improve and promote individual welfare. There are species that remain gregarious throughout ontogenetic development while others are gregarious only at the beginning of the development, becoming territorial later. Still other species are territorial from birth (Yamagishi, 1969). For example, zebrafish (*Danio rerio*) remain a gregarious species throughout life while Nile tilapia live in schools in the early stages of development and then go on to become territorial, as is characteristic of cichlids; *Betta splendens* is extremely territorial throughout life.

Despite the advantages of group life, this state of organization imposes a more or less universal cost, resulting from competition for scarce resources such as food, partners, breeding sites, and refuge sites (Huntingford, 2013). At the time of disputes over these resources, it is natural for hierarchical relationships to be established among these animals, regardless of their life habits. However, in territorial species, this hierarchy is more frequent and lasts beyond occasional disputes.

Dominance hierarchies are social structures in which dominants gain, through aggression, increased quantity or better quality of a limited resource (Huntingford, 2013). However, even for the dominant animals, these aggressive (agonistic) interactions can be energy costly and time consuming (Haller, 1991), and these fish are exposed to imminent possibility of injury (Neat et al., 1998) and increased risk of predation (Jakobsson et al., 1995). Thus, hierarchical combat requires investments based on the balance of costs and benefits (Smith and Price, 1973). Imbalances in these forces impair fish welfare.

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Behavioral basis and welfare

In the scenario of fish behavior, there is a strong body of knowledge available that has been reviewed over the last decades. In this section, will focus on the basic structure of behavior because many researchers have studied what the animals do, but rarely why they do it. This latter question requires understanding the structure of behavior. In this way, studies on fish communication (Barcellos et al., 2014; Barcellos et al., 2011; Waddell et al., 2016), social stress (Alonso et al., 2012; Ferraz and Gomes, 2009), and individual preferences (Costa et al., 2013; Malato et al., 2017) are of great importance to understand how individual fish cope with the disadvantages of grouping, especially the subordinate ones. In the context of fish welfare, adding to the interindividual interrelationships during grouping, individual behavior is also an important consideration. In the last two decades, many studies have provided more consistent and comprehensive knowledge about individual variability. The next section, on fish welfare, stresses individual preferences, and this is in line with studies of fish personalities and wants. In addition, there is one phenomenon that is not new but remains relatively understudied in fish: *anxiety*, or anxiety-like behavior. This line of investigation also provides a more holistic view about fish, a necessary consideration for the welfare issue.

Studies of anxiety in model organisms have been carried out since the 1930s. For early research in psychopharmacology and anxiety, models were used with an emphasis on learning and conditioned reflexes; in the 1950s, studies began to focus on operant conditioning (spontaneous response in animals). Several research studies have been carried out over the years using different protocols to evaluate anxiety; these protocols are classified according to the conditioned or nonconditioned responses. In the conditioned response, there is the involvement of the response to a stress; in the unconditioned response, the reactions are spontaneous—that is, they occur naturally. The most common models used are the *open field test*, which makes it possible to evaluate the anxiety of an animal through exploratory activity in an unfamiliar environment, such as an empty box; the *light-dark test*, which evaluates the behavior of the animal in an experimental box containing two compartments, one light and one dark, with the transitions made between the compartments quantified (Bourin and Hascoët, 2003); and several others. The most well-known anxiety models used in fish research (Kysil et al., 2017) are the *novel tank task* (NTT) (Giacomini et al., 2016a; Mocelin et al., 2015) and the *light-dark test* (LDT) (Mocelin et al., 2015), both of which are protocols frequently used with rodents.

The NTT is based on the geotaxis paradigm in which fish demonstrate an innate escape behavior when introduced into novel environments, a conceptual analog to the rodent open field paradigm. This test evokes motivational conflict between the protection behavior and subsequent vertical exploration. When placed in novel environments, zebrafish and jundiá (*Rhamdia quelen)* initially spent more time at the bottom of the aquarium; later, due to habituation to the novel environment, these fish gradually started exploring the top area of the aquarium (Abreu et al., 2016; Kysil et al., 2017).

For example, as expected, jundiá, in which a stressor (net chasing) induced anxiogenic-like behavior in the novel tank test, spent more time at the bottom of the aquarium in relation to the control group, as evaluated by automated tracking software (Abreu et al., 2016). This confirmed that this Neotropical fish could be used for testing anxiety-like behavior through consolidated tests and computer analyses. In addition, it has been found that the essential oils of the plants *Lippia alba* and *Aloysia triphylla* exert an anxiolytic effect on *R. quelen*, thus validating this species as a reliable model for studies on fish anxiety (Abreu et al., 2016).

Welfare

Humane treatment of animals has been a matter of debate for several centuries. The intensification of the use of breeding systems has further accentuated this problem. There is no question that humans need food to survive and no question that animal meat is part of the diet of many humans. In addition, the strategies used by humans to capture, breed, and slaughter animals are products of their highly developed intellect and therefore represent a consequence of their biological evolution. All this is accepted as a biological reality. What is questioned is the human capacity to intentionally inflict suffering on other animals, whether for the simple pleasure of seeing them suffer or due to the lack of ability or interest in dealing with them.

There are three main reasons why a human being purposefully imposes suffering on another animal: (1) insensitivity to the issue of others' suffering (lack of empathy); (2) belief that the other animal does not suffer; or (3) lack of means to prevent or minimize suffering. Of these reasons, the first is typically rejected by their common sense and cultural values; humans without empathy are labeled *sociopaths*. The second is still a topic of discussion and has been affected by many scientific and social paradigms over the centuries. The third depends on knowledge concerning appropriate means, and is heavily influenced by the second reason. In short, the questions about the well-being of nonhuman organisms are based on the question of how much these organisms can suffer. In this section, we will discuss this issue, focusing primarily on fish although the general questions apply to any animal.

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Understanding the question of fish welfare is not an isolated topic. The position of these animals relative to other vertebrates has profound implications for all vertebrates. In biological evolution, fish first appeared as amphibians, reptiles, birds, and mammals. Although each of these groups did not originate the other but shared a common ancestor, it is assumed that primitive ancestral forms were derived in different surroundings or biomes through natural selection from differential reproduction; thus, the various taxonomic groups arose. The implications of this phylogeny may be deeper, as features present in some groups may not occur in the species that preceded them. For example, effective mechanisms that maintain *homeothermia* (body temperature control, regardless of ambient temperature) only exist in birds and mammals. Thus, because fish are organisms closer to their primitive origins, they do not necessarily present all the characteristics of more evolved vertebrates. However, the existence of certain characteristics in fish suppose that these characteristics could exist in the groups that arose later (because they were not lost during the process). It is in this context that we will discuss whether the fish suffer. For example, do they feel pain? Do they suffer? Are they beings who are aware of suffering?

Here it will be demonstrated how the issue of suffering in nonhuman animals has been addressed in the scientific area. As mentioned above, if the awareness of suffering exists in fish, it may have remained in amphibians, reptiles, birds, and nonhuman mammals, placing the problem of animal suffering in a broad spectrum.

Historical rudiments of considerations about animal feelings

Contrary to the idea of most Renaissance artists' acceptance of emotions in nonhuman animals, Descartes' seventeenth-century dualist view held that only humans possessed emotions, ascribing to other animals life without emotions and feeling, or true automata. This dualistic view, that the organism is composed of body and mind as distinct entities, was greatly reinforced by religious precepts that attributed this "something more" only to humans. According to certain religions, although all creations are divine, only human beings were created in the likeness of a God and had reason. This belief divides animals into rational and irrational categories, a misconception that continues to be taught erroneously in some schools or areas in different phases of education.

In the eighteenth century, the positions of philosophers Jeremy Bentham and David Hume returned to the suggestion of feelings in nonhuman animals. Accepting these feelings was not difficult, as the interaction of people with domestic animals brought information that reinforced emotional states in animals (a happy dog wagging its tail for its human companion). This vision continued into the nineteenth century where people encountered veterinarian William Youath, who wrote about horses, oxen, sheep, pigs, and dogs and admitted the existence of emotions in these animals.

The evolutionary theory advocated by Charles Darwin at the end of the nineteenth century was another important milestone in the argument that nonhuman animals are sentient beings. In this theory, continuity can be traced among living beings, making it less logical from a religious perspective that only humans would be created in the image of their god. All existing organisms would be successful forms of a continuous process of natural selection, for which the existence of adaptive mechanisms is fundamental. Thus, subjective elements, such as feelings, would be seen as adaptations critical to the struggle for survival (Yamamoto and Volpato, 2007). In spite of this scientific reinforcement of the popular belief in emotions in nonhuman animals, the early twentieth century returned to behaviorist psychological views, arguing that nonhuman organisms are automata beings guided exclusively by responses "blind" to stimuli through conditioning.

The question of well-being remained obscure for decades, mainly due to World War II. In the late 1950s and early 1960s, it again became a topic of attention, focusing on animals used for food (Welty, 2007) and biomedical experimentation (Rikleen, 1978). However, the book at the foundation of the history of the science of welfare was Ruth Harrison's *Animal Machines* (1964), which led to a follow-up investigation and the Brambell Report of British Government Investigations, with the results published in the form of Command Paper No. 2836 (1965). In December 1979, the British Council for the Protection of Farm Animals defined welfare based on the "Five Liberties" for farm animals (cattle, pigs, sheep, and chickens), which stated that these animals should be free from (1) thirst, hunger, or malnutrition; (2) discomfort; (3) pain, injury, and illness; (4) impediments to express most normal behaviors; and (5) fear and pain. Although these Five Freedoms originated with the 1965 Brambell report, they also reflected the essential human freedoms expressed by US President Franklin Roosevelt in 1941. The criteria, however, lacked objectivity and therefore a definition of criteria for emotional well-being (Volpato et al., 2009b).

Since then, several studies have emerged on the well-being of nonhuman organisms. However, in the late 1990s and particularly at the beginning of the twenty-first century, numerous studies have emerged as the product of intense and frequent discussions on animal welfare. From these debates, special volumes of scientific journals dealing with animal welfare appeared more systematically from the second half of the first decade of the present century. In relation to fish, two journals dedicated a special issue to the theme (Diseases of Aquatic Organisms, Vol. 75, 2007, and the Institute of Laboratory

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Animal Research's *ILAR Journal*, Vol. 50, 2009). In addition, multiple articles have appeared in the literature of this century, particularly in this decade.

In the course of the brief history of animal welfare, recent issues have basically centered on four aspects: (1) the extent to which nonhuman animals, including fish, deserve to be considered as beings that can suffer (i.e., sentient beings); (2) how to evaluate the welfare of these animals; (3) how to maintain the zootechnical production of these animals while imposing on them only minimal suffering; and (4) how to maintain experimentation with animal models while safeguarding welfare issues for experimental animals.

Reasons for debates on fish welfare

One of the defenses of practitioners who impair the well-being of fish is that these animals are not aware of the pain—that is, they are not sentient beings. Of course, pain is one of the important elements in the welfare issue, but not the only issue. For example, the discomfort in the animal should be avoided, and discomfort includes but is not limited to pain (Volpato, 2000). Conditions that do not cause pain but that nevertheless cause discomfort are delayed food and water, inadequate temperature, absence of a resting place, excessive lighting, lack of circadian periodicity of light, alert stimuli, the presence or perception of predators, high population density, poor water quality, reduced space, presence of a dominant species, and the lack of the possibility of expressing themselves according to their own desires (e.g., movement, vocalizing, etc.).

However, as a precursor to discussions about the welfare of these animals, it is necessary to understand that not only are there conditions that cause the animals discomfort, but that the humans responsible for causing these conditions are aware of this discomfort. It is at this point that the question of well-being in nonhuman organisms becomes more complex. Emotion triggers unconscious visceral, behavioral, hormonal, and neural responses to stimuli and aversive or attractive situations. Emotions are the backdrop to the onset of feeling, which results from further processing at conscious levels in the higher cortical regions. Thus, emotion is a prerequisite for feeling, which operates on a conscious level.

Many complex activities can develop at nonconscious levels. For example, when people are driving a car, they can talk to the other person next to them, listen, sing a song, and think about activities and things in their life. As this happens, people continue to drive and often realize only later that they do not remember every detail of events occurring along the steering path. We brake, accelerate, slow down, signal, check the mirrors, and turn, all without realizing it, while concentrating on an additional activity such as conversation. This shows that our brain can process complex information from the environment and direct our behavior without our being fully aware of it. The conscious state can be directed to one focus while other activities take place simultaneously in the background. Thus, the act of performing processing complex activities is not an unequivocal requirement of the state of consciousness.

Among the states of consciousness, the most rudimentary is sentience—the capacity by which organisms perceive and distinguish internal states of pleasure and displeasure, heat and cold, comfort and discomfort, and pain. Pain is the conscious perception of nociception (a physiological sensory response of neurons perceiving noxious stimuli). Thus, to consider the well-being of an organism, that organism must be sentient—that is, it must present conscious awareness of states of pain and therefore be able to suffer. If this state exists, even if there is no pain, there exists the possibility of suffering for other reasons. For example, there may be no pain at a particular moment, but there might be hunger, anxiety, or some form of discomfort. The leap between the physiological perception of stimuli and situations and the generation of states of consciousness (such as suffering) requires a neural basis that, at least in humans, is attributed to the neocortex.

Despite the links between mental states and the anatomical and physiological bases of the brain, the confirmation of conscious states, even rudimentary sentience, in nonhuman animals is fraught with problems. In a review (Yamamoto and Volpato, 2007), the fallacious logic of such attempts was analyzed, the main points of which are summarized below.

Volpato et al. (2007) showed the difficulty of people accepting sentience in animals phylogenetically distant from humans based on the following argument. People are sure of their own existence and consciousness. From there and from the verbal information received from other people, they consider that these mental states occur in all human beings. When it comes to newborns, who communicate little with them, knowing whether infants have states of consciousness about themselves or are suffering could be more problematic. However, by simple inference, people admit that the brain of a newborn works very much like an older child or even an adult. This thought, which brings them an analogy by similarity, would lead them to accept that the more phylogenetically distant the animals are in relation to the human species, the more reckless it is to assume that they have the more complex brain functions and capacities that humans experience in their being. This is just an inference, but it is similar to what humans do when they consider suffering in newborns. They present expressions (motor or not) that humans interpret as joy, feeling pain, suffering, or discomfort. However, notice that there is a distance between our feeling, what people say about it, or the speech they hear from others about their possible feelings. Given the high sophistication of human expression, people are easily led to accept that they are discussing the same psychological

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phenomenon when they talk with other human beings. It is from this experience that people most readily accept certain emotional states in newborns; however obvious it may be, this involves some degree of extrapolation. Nevertheless, this has been questioned.

The further away phylogenetically the species are from humans, the more likely it is that these "sophisticated" conscious states do not exist. In general, people accept awareness in a monkey or a dog, but they have difficulty accepting this in a bird, or for a reptile, amphibian, or fish, let alone for invertebrates. Thus, the central issue for the validity of welfare concerns in nonhuman animals, particularly for fish and others phylogenetically closest to them, is the proof of sentience in these animals.

How has sentience been studied in fish?

Within the difficult debate evidenced above, considerable research has been developed in the search for evidence that fish are sentient beings and therefore capable of suffering some degree of discomfort, which would validate concerns about their well-being. The results of these studies are very convincing of this ability in fish, but they are far from conclusive. Even so, empirical evidence can be used as an indirect assumption in the argument about fish welfare.

An approach to fish sentience as well as other mental states seeks to analyze the cerebral morphological basis of these states and to identify it in animals. Thus, brain areas such as the limbic system and cortex are important. However, these areas do not occur in all vertebrates, let alone in all animals, which, according to this view, suggests that these more primitive organisms do not have neural bases that support states of consciousness. In this regard, the study by (Rose, 2002) was a landmark, as it showed that in fish, there is no connection of the nociceptive sensory pathways with higher cerebral centers that signify awareness of these sensations. Such an approach presupposes that the neural substrate referent of mammals, or humans, is the primary reference for studying animal consciousness. In a move contrary to that proposed by (Rose, 2002), studies initiated by Victoria Braithwaite of the University of Glasgow in Scotland tried to show the neural base of pain in fish. Part of these studies was led by Lynne Sneddon, who, at the beginning of the century, represented a strong initiative against Rose's studies. The demonstration of nociceptive nerve pathways (responding to thermal, mechanical, and chemical stimuli) in fish, together with the observation that they are mammal-like fibers (Sneddon, 2002; Sneddon et al., 2003a), reinforces the idea that these animals perceive painful stimuli. However, as Rose (Rose, 2007) argues, nociception of pain does not necessarily imply that the organism perceives it on a conscious level.

There have also been behavioral studies seeking to demonstrate that fish feel pain. In this sense, the most classic was that of Sneddon et al. (2003a). She studied rainbow trout (*Oncorhynchus mykiss*), injecting acetic acid (which is known to induce pain in mammals) or bee venom (also inducing pain) in the region of the lips (where there are several pain-sensitive fibers). Unlike trout that did not receive these substances, the trout with the painful stimulus increased ventilation of their gills, took longer to feed again, and rubbed their mouths on a substrate. These behaviors strongly suggest that these trout were reacting to the pain caused by the injection of acetic acid or bee venom, but did not demonstrate actual *awareness* of the suffering. Moreover, these behaviors did not occur and the trout behaved as if nothing had happened when they received, before the painful stimulus, a morphine injection. Morphine is recognized as a potent analgesic, which favors the interpretation that the trout did not feel the pain. Morphine is an opioid that acts on specific receptors that naturally exist in the body, so this also showed that fish have opioid receptors. The implication is that mammalian opioid receptors are important for endorphins and enkephalins, which are endogenous substances that modulate pain. Now, would fish naturally have receptors for endogenous substances that reduce pain if they cannot feel pain? However, there is no unconstrained empirical demonstration of consciousness about pain.

There is a recent body of literature focusing on pain in fish, specifically using the zebrafish because this species possesses robust nociceptive responses and pain receptors (Currie, 2014). Given the fundamental role of the opioid system in pain control, the study of the zebrafish opioid system is essential for our understanding of pain pathobiology (Demin et al., 2018). Both adult and larvae zebrafish display overt behavioral responses to nociception, similar to those in mammals. Among the behavioral changes, algogens cause hypoactivity, which can be assessed easily using automated video-tracking recordings (Kalueff et al., 2013). Zebrafish also provide excellent, translationally relevant genetic tools for studying pain, including mutant lines identified in forward mutagenesis screening and reverse genetics for specific genes, including delta-opioid receptor genes (Barrallo et al., 1998), mu-opioid receptor genes (Barrallo et al., 2000), and kappa-opioid receptor genes (Alvarez et al., 2006). In addition, all these genes (delta, mu, and kappa) are highly homologous to the respective human genes (Gonzalez-Nunez and Rodriguez, 2009). Thus, zebrafish studies using an array of noxious stimuli, administration routes, and pharmacological manipulations support the notion that the fish feel pain and are a viable model in the study of pain pathobiology (Demin et al., 2018).

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Even here, skeptical criticism still finds its way. Physiologically perceiving pain (having pain receptors stimulated) does not necessarily imply that a sensation evokes a conscious awareness of pain. The responses triggered in the fish may be subconscious, indicating only nociception (physiological perception of the painful stimulus) without this reaching higher brain levels. Therefore, more sophisticated studies have sought to show that fish do have complex cognitive abilities and therefore should be considered sentient beings. Not only can fish be conditioned, but they can also form cognitive maps of their environments, and they have been shown to evaluate notions of time in conditioning studies. Braithwaite's studies support the existence of more sophisticated cognitive levels in fish (see Braithwaite and Boulcott, 2007; Sneddon et al., 2003b). A study by Portavella et al. (Portavella et al., 2002) shows that fish have brain structures homologous to the mammalian amygdala and hippocampus, two brain regions involved in mammalian emotional processes. Changes in these structures impair the ability of fish to learn more complex tasks. Sneddon (2006) studied the brain of the common carp using magnetic resonance imaging and detected significant activity in the forebrain, followed by activities in the midbrain and the hindbrain during painful stimulation. In carp and trout, this study also showed an increase in gene expression in these brain regions after painful stimulation. These results clearly challenge Rose's argument about the disconnection between peripheral nociceptive fibers and brain structures in fish.

In the following section, the logic of these proposals is analyzed, showing that the search for proof of sentience and other levels of consciousness in nonhuman animals goes beyond the methodological resources of empirical science. However, there are sufficient reasons to consider the welfare of these animals.

Logical reasons for the fish suffering issue

Empirical science seeks to validate its conclusions through observable evidence (Volpato, 2007a, b, 2010a, b). With this analytical bias, the study of consciousness is a difficult task, even in humans, let alone in nonhuman animals. This difficulty has long been recognized in science (Dawkins, 2006; Duncan, 2006; Hastein et al., 2005; Rushen, 2003; Sandøe et al., 2004). However, the difficulty lies in the relation to the object of study (consciousness) because it is an entity that cannot be directly registered. Operational variables derived from the theoretical variable consciousness are not, and could not be, absolute to deal with consciousness.

This inability of science, however, has been used strangely by those who seek to study the welfare of fish and other nonhuman animals with the preconception that they are *not* sentient beings. These people claim that it has not yet been scientifically demonstrated that these animals do suffer or feel pain or that either of these occurs on a conscious level. "Of course not!" they say. Their preconception allows them to maintain practices that impose pain or discomfort on these animals, such as sport fishing and the use of live bait. However, a deeper analysis shows how such an attitude is inappropriate. Science works with direct evidence and indirect evidence; this is inevitable. More recurrent approaches to seventeenth-century science still rely on the belief that the data are objective enough for all scientific studies. However, they forget that such a high standard has long since been abandoned, even within the area of law. Indirect evidence can be used once the context of the analysis supports its adequacy. Not every criminal leaves explicit clues and this needs to be dealt with.

Another way to address the dilemma of the inability of fact-based science to address conclusively issues of consciousness and emotion in nonhuman animals is this: simply reverse the burden of proof. Why is it necessary to prove that fish are sentient to treat them accordingly? Why not prove that they are not sentient before treating them without welfare considerations? This is the central point! In three reviews on the subject, Volpato and several other authors have advocated the need for this reversal of the burden of proof (Volpato et al., 2009a, b; Yamamoto and Volpato, 2007). If empirical science cannot objectively and directly assess the problem of conscious suffering in fish and other nonhuman animals, then it also fails to say that these states do *not* occur in these animals. It is not a question of first proving that fish suffer and then taking care of their well-being. If it is not possible to prove that fish do not suffer, it is reasonable that they should not be subject to practices that would lead a sentient being to suffer. This is the simplest logic in the matter. In democratic societies, people are not accused of crimes and then burdened with the task of proving their innocence; rather, what is required is that the prosecution must provide evidence that proves their guilt. Thus, if people want to call themselves good for returning fish to the water after they catch them while they seek to feed themselves, one must first prove that they do not suffer. That is, it was insisted that the burden of proof remain with the prosecution. This is especially valid given that the possibility of conscious suffering is inherent in these beings.

Substantiating this view, the massive concentration of biological data is nothing more than a confirmation that nonhuman animals, among them fish, can suffer. In addition to the logical questions posed above, there is still data suggestive of the various scientific studies emphasizing that fish are sentient beings and therefore deserve to be treated without suffering. Additionally, the suffering involves more than pain, as it can involve even the fact that the animal cannot behave

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freely. Consider the wide range of revisions on the subject: (Balon, 2000; Bekoff, 2008; Broom, 2008; Brydges and Braithwaite, 2008; Dawkins, 2006, 2008; Duncan, 2006; Fraser, 2008; Jennings, 1998; Lehman, 1998; Pelletier et al., 2007; Takahashi-Omoe and Omoe, 2008; Toni et al., 2019; Veissier et al., 2008; Veissier and Forkman, 2008; Veldhuizen et al., 2018; Volpato, 2009; Volpato et al., 2009b; Volpato et al., 2007). Critics of these views are James Rose (United States), mainly on the issue of animal pain, and Robert Arlinghaus (Germany) (e.g., Arlinghaus et al., 2007, 2008, 2009) on sport fishing.

How to evaluate fish welfare?

An increasing attempt to consider well-being has been through studies of stress. Many studies have simply replaced the focus on "stress" with "well-being." However, stress and well-being are not antonyms. The stress state is an adaptive response that enables the body to overcome adverse conditions. In stress, there is sympathetic stimulation, which prepares the animal for emergencies, as well as mobilization of substrates that supply it with sufficient energy. As shown in Volpato et al. (2009b), people can clearly distinguish the state of disease and the state of health. In this case, stress is present, both in disease states and in health. In addition, in the proposed scheme (Volpato et al., 2009b), distress may also be in health and disease conditions. From all this, is possible to conclude that the welfare state occurs in healthy organisms, whether they are in states of stress and distress.

In the case of stress, neural and neuroendocrine mechanisms are clearly defined in all vertebrates. This is because it is a condition that allows natural selection (Volpato et al., 2009b; Yamamoto and Volpato, 2007). For example, stress is an internal state that occurs when the homeostasis of the individual is threatened. With this, the body triggers a series of responses that usually culminates in the assurance of energy and emotional and perceptive capacities to face the danger. This set of responses is mainly mediated by two physiological systems: (1) the sympathetic nervous system, which stimulates the adrenal gland to produce and release catecholamines (adrenaline and noradrenaline) and (2) the hypothalamic-pituitary-adrenal system, which is a cascade of neural and hormonal events that culminates in the release of glucocorticoids from the adrenal cortex (in fish they are the interrenal cells), constituting the hypothalamus-pituitary-interrenal (HPI) axis, homologous to the hypothalamus-pituitary-adrenal (HPA) in mammals. The catecholamines fail to penetrate the brain, and the release of noradrenaline occurs in this tissue, occurring from the nucleus locus coeruleus (Morilak et al., 2005), which is involved in states of alertness, vigilance, and attention. Throughout biological evolution, these physiological processes of stress were selected because they enable organisms to face challenges and survive to reproduce. Hence, stress triggers a relatively well-standardized set of physiological responses, the result of natural selection acting to balance the advantages of stress responses with the stress context for the lives of these animals.

It is important to consider that the occurrence of potentially stressful situations triggers more immediate stress responses, mediated by the strictly neural axis of the response system (motor responses of alert and even neurovegetative responses that may also arise from the release of the hormone adrenaline). Responses involving the activation of the neuroendocrine axis of stress take, on average, 15 min to peak. This shows that the stress response involves both neural and neuroendocrine triggering, culminating in motor or vegetative behavioral adjustments. Interesting results have indicated that fish could receive visual cues of a large but physically separated heterospecific predator without triggering the classic whole-body cortisol response, but that chemical cues through conditioning (water transferred by a predator-stressed conspecific fish) immediately triggered a series of behavioral motor behavior-related responses consistent with avoidance of the possible risk situation (Barcellos et al., 2014; Oliveira et al., 2017). These studies suggest an internal modulation that leads to a hierarchy of response levels to be triggered by the hypothalamus based on the degree of the risk stressors.

In the case of welfare states, the same pattern does not occur. The individual can be in conditions of well-being in situations of great energy expenditure, or even in those of complete rest. This disparity of possibilities prevents all responses from being consummated by a single, or very few, motor or neurovegetative mechanism that may have been evolutionarily selected. For this reason, Volpato et al. (2007) argue that the search for physiological mechanisms of well-being should be replaced by studies to understand the conditions under which animals are in a state of well-being.

Dawkins (2006), Duncan (2006), and Volpato et al. (2009a, b) have warned people about how important it is to pay close attention to what animals have to tell them. Of course, people need to find appropriate forms of investigation to understand what animals are telling them. A practical way to solve this problem is through preference tests, which were already used for this type of problem but were somewhat forgotten, given the emphasis on understanding the physiological bases of sentience in nonhuman animals (Yamamoto and Volpato, 2007). The following excerpt from a paper by Marian Stamp Dawkins explains this well.

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> *There is a danger that well-meaning people define animal welfare in terms of what they think animals want or what pleases them. But if we take animal sentience seriously, we must ensure that the animal voice is heard […]. We now have […] methods for "asking" animals what they want and we should […] use this evidence […] and ask the animals rather than automatically assuming that we know from our human standpoint. Animals are not […] looking at the world through human eyes and science can help us […] to look through those different eyes. Real respect for animals will come when we see them as sentient beings in their own right, with their own views and opinions, their own likes and dislikes. The animal voice should be heard.*
>
> (Dawkins, 2006)

Like this, it is necessary to know the conditions of preference of the animals to be able to use them in cases where people keep these animals in captivity. Environmental enrichment cannot be achieved when perceived through the eyes of humans, but only through the animals' own perceptions of their needs. Thus, well-being can be defined as the internal state of the animals when they are in conditions in which they freely chose to be (Yamamoto and Volpato, 2007). Based on this, and emphasizing Dawkins's (Dawkins, 2006) proposal, the use of preference tests and a series of precautions should be taken when applying these tests to fish.

Preference studies are quite useful in ambience questions. For example, fish living in shoals in their natural environment experience isolation as a source of stress and anxiety because aggregation in schooling represents an important form of socialization that reduces the risk of predation (Miller and Gerlai, 2007). For these fish, laboratory studies do not reproduce the form of socialization in schools because many experimental protocols are carried out with individually housed fish, thus breaking up innate strategies of schooling in these species (Pagnussat et al., 2013). With this, the response to stress can be compromised and can interfere in the results, which might explain the physiological, neuroendocrine, and behavioral mechanisms of defense due to the deprivation of these animals of signaling among the cospecific animals. This may create an artificial set of responses of academic merit but of little biological validity. The isolation depresses the reactions of such fish to the environment, reducing their capacity to respond to stressors (Giacomini et al., 2015). Thus, keeping fish of gregarious behavior in situations of isolation constitutes a strong factor of loss to the degree of well-being. Likewise, grouping species of isolated habit may impose on those animals stimuli that exacerbate the frequency of agonistic interactions, causing physical and/or psychological damage to individual fish.

Still, in relation to the ambience, fish have preferences for certain colors and the presence of refuges, which suggests that people must take care with the artificial environments in which they maintain them. It is known that the color of the light and the walls of the tanks influence the stress responses in fish of different species such as *O. niloticus* tilapia (Volpato and Barreto, 2001), *Pagrus pagrus* (Rotllant et al., 2003), and jundiá (Barcellos et al., 2009), and also affect the reproduction of *O. niloticus* tilapia (Volpato et al., 2004). The color of light, for example, blue light, also affects the behavior of various species of fish (Fanta, 1995; Kawamoto and Takaeda, 1951; Loukashkin and Grant, 1959). Green is reported as a color of well-being in matrinxã *Brycon cephalus* (Volpato, 2000) and sardine *Sardinops caerulea* (Loukashkin and Grant, 1959). It is also known that in addition to the color of the light and the walls of the tanks, the presence of an adequate number of dark refuges may represent better welfare conditions, especially for nocturnal fish species such as jundiá (Barcellos et al., 2009).

Related to ambience and preferences is the idea of environmental enrichment. Over the years, there has been increasing concern about the welfare of animals raised in captivity and animals used for research and production. According to the agencies regulating animal welfare and research procedures (Parliament, 2010), respecting the old Brambell report, animals should be kept in an environment with space and complexity that allows expression of their normal behaviors. In addition, numerous studies have been carried out using enriched environments in behavioral experiments on fish (Collymore et al., 2015; Lema et al., 2015; Manuel et al., 2015; Nijman and Heuts, 2000; Schroeder et al., 2014). However, in aquatic species, there are few reports on endocrine and behavioral responses in enriched environments. In fish, environmental enrichment increases the rate of cell proliferation in the telencephalon (Lema et al., 2015) and zebrafish (von Krogh et al., 2010) while also increasing the recovery and adverse effects caused by stressors (Pounder et al., 2016) and by residents' ability to establish dominance over intruding fish (Nijman and Heuts, 2000). (Schroeder et al., 2014) have shown that zebrafish prefer enriched environments without the complexity of the natural habitat, and this preference may vary according to the fish accommodation forms (Collymore et al., 2015).

Studies on the benefits of environmental enrichment in promoting animal welfare are helpful in assessing responses to stressors to which fish are constantly exposed, not only in the natural environment but also in manipulations imposed in research laboratories and aquaculture. Recently, it has been shown that the difference in cortisol concentrations between zebrafish kept in isolation and those in groupings can be controlled through environmental enrichment (Giacomini et al., 2016b). In addition, that study also showed that environmental enrichment might inhibit the classic response of cortisol elevation in zebrafish that are isolated and stressed. The authors hypothesized that such a reduction in the stress response results from the fact that environmental enrichment gives fish a sense of security because they are in a natural-feeling

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environment, including refuge alternatives. Similarly, maintaining zebrafish in an enriched environment for 21 or 28 days promotes positive effects when the animals are subjected to unpredictable chronic stress (Marcon et al., 2018). The anxiolytic effect of environmental enrichment has been demonstrated in some species, such as rodents (Hajheidari et al., 2015; Ragu Varman and Rajan, 2015) and fish (Manuel et al., 2015). Along these lines, some studies have shown a relationship between environmental enrichment and serotonin. In fact, environmental enrichment increases the expression of 5-HT receptors in mice (Ragu Varman and Rajan, 2015) and reduces painful sensations by increasing the pain threshold (Pham et al., 2010; Vachon-Presseau et al., 2013).

Auditory (musical) environmental enrichment can also be used to improve the welfare of laboratory animals, with clear positive behavioral effects and general stress relief in several species, including dogs, primates, pigs, horses, and rodents (Alworth and Buerkle, 2013). In contrast, chronic exposure to loud noise in laboratories may impair the well-being of experimental animals (Patterson-Kane and Farnworth, 2006). The exposure of classical music to production tanks increases productivity in several species of fish (Catli et al., 2015; Imanpoor et al., 2011; Papoutsoglou et al., 2007; Papoutsoglou et al., 2010), modulating positively physiological and metabolic states (Papoutsoglou et al., 2010).

The reaction of fish to music has also been investigated, as they are animals that can hear sounds in the aquatic environment (Popper and Fay, 2011). For example, exposed to classical music in culture ponds, carp (*Cyprinus carpio*) (Papoutsoglou et al., 2007) and turbots (*Scophthalmus maximus*) (Catli et al., 2015) grew and fed more efficiently. Recently, (Barcellos et al., 2018) showed that environmental enrichment with exposure to Vivaldi's music made zebrafish less anxious and more active. In addition, when compared to the control without background music, the exposed group did not present evident responses to stress, either by cortisol analysis in the whole body or in the unchanged expression of CNS genes related to the stress response. These studies demonstrate that environmental enrichment is an alternative neuromodulatory strategy to reduce the behavioral and physiological impacts of stress and may result in an increase in the level of well-being of fish and other animals under human-imposed conditions of confinement. Even with these considerations, Ellis et al. (2012) emphasized the need for further studies on catecholamine responses in stress situations. In addition, they stated that more studies are needed to know the exact role of these responses on the mood of animals, thus achieving greater value for welfare issues.

Regardless of the contributions that stress studies bring to the understanding of well-being, there is still a very large gap for physiologically understanding such a state. Conditions of positive well-being may be associated with the presence of stress. According to Koolhaas et al. (2011), there are several situations that include cortisol elevation, such as during reproduction, that people should be wary of interpreting as poor well-being. When captive animals are challenged with obstacles to feeding themselves (e.g., meat within blocks of ice offered to bears in zoos), they probably are in a stressful situation, causing cortisol elevation in these animals, and such challenges can be seen as important for maintaining good welfare. Thus, it is always important to discern stress from distress, the latter being the state of "stress" at the level that compromises some necessary biological processes (e.g., immune response, growth, reproduction). Therefore, physiological analyses of stress should not be conducted in isolation, but in consideration of the context in which they are manifested so that they can be used adequately in the science of well-being (Volpato et al., 2009a).

The personality of fish and their well-being

Temperament can affect the ways in which animals interact with the environment, predators, food sources, and social or sexual interactions with members of the same species (Réale et al., 2007). Temperament is often associated with responses to new, risky, or challenging situations (Wilson et al., 1994). This is evidenced through tests on its repeatability over time and maintenance with great independence in relation to environmental context. Despite this, there are cases where the answers can be consistent over time but not in different contexts—in this case, not being considered as personality traits. Preferences, more or less consistent, certainly affect how animals interact with their environmental resources and also with the challenges imposed in these environments. Thus, understanding the differences between individualized behaviors (i.e., temperaments, personalities) facilitates understanding of fish reactions to environmental stimuli. This is particularly useful for maintenance in research laboratories or even for aquarium situations where fish can be maintained in isolation or in limited numbers. In such cases, individual expressions should be considered.

The most studied behavioral continuum in fish is the "shy x bold" variation. In this continuum, bold fish usually present high exploratory activity in relation to new objects, with a reduction of anxiety-like behaviors and changes in body color (Oliveira et al., 2015; Réale et al., 2007). In contrast, shy fish exhibit low exploratory activity and increased reactivity to novelty and risk (Kalueff et al., 2013). Other studies have investigated the genetic basis of such "hesitant and daring" behavioral patterns and have shown that the daring phenotype in zebrafish may present an evolutionary genetic advantage (Oswald et al., 2013).

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Furthermore, the personality traits or temperament are directly related to the immune system of fish. Of course, fish with different patterns of exploratory behavior present important differences in their immunological profiles. Fish that do not explore a new object in a test aquarium present a proinflammatory profile compared to fish that do explore the new object (Kirsten et al., 2018a, b). In addition, diseased fish also present reduced exploratory behavior. This reduction of exploration of a new object can be interpreted as a defense mechanism because it reduces the risk of predation and exposure to pathogens, although it may in some cases be a mere consequence of the inability of the animal to express itself more freely or spontaneously (e.g., in the case of diseases). In addition, in diseased fish, the activation of the immune response is also associated with reduced social preference, and a diseased zebrafish loses its innate preference to be close to its cospecific cohort. Whatever the most immediate cause of isolation is in a disease situation, this behavior is a hallmark of several species, possibly as a consequence of the reduction in the possibility of dissemination of pathogens that could result from aggregation (Kirsten et al., 2018a, b).

Thus, the behavioral complexity of the fish becomes increasingly clear and the study of personalities becomes important in the context of well-being. The relationship of these personality issues with the immune system points to the fact that the maintenance of fish of different personality patterns in tanks with the same configuration and management may imply poor well-being of this or that group. Fish, whether neophobic or neophilic or bold or shy, have distinct needs, and therefore their welfare requirements must be respected within their respective temperament or personality classes.

The preference tests as indicators of conditions for well-being

The above ideas reinforce proposals that seek to indicate to animals, in a less punctual and more holistic context, their moments of well-being. There are no reliable tools for identifying well-being or welfare conditions by physiological resources. Physiological or behavioral stress responses may not be unmistakable synonyms of good welfare. That is, the absence of any physiological stress response (e.g., low cortisol levels) may not be indicative of a state of well-being but instead an inability to deal with stressors. In cases of distress, it is easier to consider conditions of poor welfare. However, even there we must be cautious. Fish in open migration could be said to be in conditions of poor well-being. At the immediate physiological level, they struggle and weaken; some die. Nevertheless, would that imply that simply giving up the struggle (reproduction) that would lead to passing on their genes (obviously an unconscious struggle) would induce a state of well-being? Is it necessary to assume that what animals do naturally (as in the analysis of stereotyped behaviors) is part of their welfare state? Securing reproductive partners, territories, or resources are energy-demanding activities and thus certainly related to high levels of cortisol and other indicators of well-being, and these are all within the expected repertoire of various animal species. Would this be a state of poor welfare, even considering that these are steps necessary for the best adjustment or adaptation of individuals and species?

On the other hand, in other contexts, detection of states of distress (e.g., the effects of environmental pollution, predation that does not culminate in death, assault with injuries) can clearly indicate states of poor welfare. In addition, generally practices aimed at assessing the states of well-being of animals in confinement usually describe a present condition, as in the diagnosis of a disease, which helps to change the conditions of the animal. However, preventive actions are even more necessary. The ideal situation is to know what leads animals to seek and preserve conditions of well-being, such as preventive health. That is, it reinforces the quest to know what animals prefer (as alluded to by Dawkins, 2006, 2008) and in which contexts these preferences manifest themselves. As supported by Volpato (2007a, b), except in pathological cases, animals choose something that immediately benefits them, although this may not prevent losses in the medium or long term.

Most of the studies in the field use tests of choice to detect the preferences of animals (Gonçalves and Oliveira, 2003; Schlupp et al., 1999; Webster and Hart, 2004). However, the effectiveness of choice tests in representing animal preferences has been immersed in methodological controversies. Seeking a solution, Maia and Volpato (2016) introduced a history-based method to analyze fish choices, but also one that would be valid for choices in other animals. The method makes it possible to discriminate between momentary choices and more consistent preferences over time. Moreover, the history-based method identifies the intensity of the preferences, including detecting nonpreferred features. Maia and Volpato (2016) showed that the most preferred items identified by this method are those to which the animals dedicate the most physical effort (Maia and Volpato, 2017) and psychological effort (Maia et al., 2017) to obtain. This corroborated that their results represented the preferences of the animals.

The history-based method recommends that tests of choice be made across several days, usually more than four, consecutive or not. Among these tests, the most recent tests carry more weight in the calculations but older tests are never discarded because it is the comprehensive view that gives the best answers about the welfare of the animals. Thus, it is the conjunction of well-being and welfare. As internal motivation and development may change over time, Maia and

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Volpato (2017) assume that, in the context of animal welfare, animals have variable desires in addition to their innate needs. Thus, by detecting preferences to give the animals a better environment, they likewise assume that animal preferences may change over the course of testing. For these reasons, preferences are best determined by cumulative sets of tests. Therefore, the proposed method is based on the "historical" record of a consecutive set of tests that gives more weight to the most recent results but does not ignore the older results. This method allows the researchers to sporadically perform subsequent testing by entering previous data into the method and adjusting the current results.

Concluding remarks

Briefly, scientific studies strongly suggest that fish are sentient beings, capable of suffering in adverse conditions. Whereas empirical science cannot demonstrate unequivocally that this mental state exists in fish, it also fails to show that it does not. Therefore, as a measure of caution, the most ethical alternative is to assume that fish are sentient beings until proven otherwise, placing the burden of proof on detractors whose ideas run counter to the most solid body of scientific evidence today.

With these considerations, the basic proposal is that any animal, including fish, must have mechanisms that enable it to distinguish between good and bad conditions. Thus, abusive practices that can cause fish to suffer, such as sport fishing, the use of fish as live bait, poor treatment of animals in captivity (which prevents them from escaping, avoiding adverse conditions, or seeking more comfortable conditions) should be urgently abolished, despite the enormous sums of money and profits related to these practices. The focus should be mainly on those activities that cause suffering or discomfort in animals and whose product is simply sport or human pleasure (after all, there are many other ways to have fun). Although it has been said that playfulness is an evolutionarily acquired human trait, rationality is as well, and it can be used to lead more humane positions in such judgments. This does not automatically preclude the use of animals for the sustenance of the human population, either as food or as part of the sound and necessary scientific research that enhances the depth and breadth of scientific knowledge and prepares us to face future adversities. A more complete review of the implications of fish welfare considerations for scientific research, teaching, pisciculture, and fishing can be seen in Volpato (2009). Even though such activities may cause discomfort and suffering to animals, good practices in science sometimes require hard choices to be made that behoove us to ensure the least possible levels of discomfort and suffering of the animals under human responsibility—and to be absolutely certain, given the ethical considerations of causing such suffering to sentient beings, that such activities are justified and ineluctable.

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Chapter 5

Stress and immune system in fish

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Introduction

The current knowledge about an animal's complex manifestation to a threatening environment, collectively called stress, has received important contributions since the 19th century. That's when Claude Bernard introduced a theory about the existence of complex mechanisms of stabilization of the internal body environment in living organisms, which allowed them to adjust to external changes. In the early 1900s, Walter Bradford Cannon extended Claude Bernard's concept of the internal environment and created the term "homeostasis," defined as a property of living beings that enables them to regulate their internal environment to maintain a steady status through adjustments to their dynamic equilibrium by physiological mechanisms of regulation (Cannon, 1929). Cannon suggested that rapid activation of the sympathetic-adrenal system preserves the internal environment, promoting compensatory and anticipatory adjustments to increase the probability of the animal's survival. He anticipated the existence of neuroendocrine responses and ingeniously detected the release of a hormone called "adrenin" from the adrenal medulla gland in the blood in response to an animal's exposure to cold. Cannon also showed that both physical and emotional disturbances triggered the same body responses and that there was a critical level of stress in terms of intensity and duration from which the regulatory mechanisms fail and the organisms perish (Cannon, 1939). Influenced by Cannon, Hans Selye established the concept of General Adaptation Syndrome, according to which the stress response presents different stages, such as a rapid reaction of alarm, through the immediate activation of the sympathetic-adrenomedullary system. This is followed by a more prolonged reaction of adaptation and resistance through the activation of the hypothalamic-pituitary-adrenal axis, and, finally, a terminal stage of exhaustion and even death, depending on the chronicity of the response (Selye, 1936).

In recent years, a broader view of the concept of stress that was proposed by Hans Selye has been discussed to accommodate the heterogeneity of stress responses that varies according to several factors, including individuals, species, populations, evolutionary history, parental effects, physiological conditions, behavioral phenotypes, and environmental effects (Schreck and Tort, 2016; Balasch and Tort, 2019). There is still little understanding of how the severity and extent of stress modulate response patterns and the boundaries between stressors to which animals can cope and adapt and those that are maladaptive.

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Teleost fish, like other animals, are constantly and regularly submitted to external and internal environmental changes, against which they trigger compensatory and adaptive responses. However, in rearing environments, fish also face unexpected threats that, depending on their origin, intensity, and duration, overcome their ability for tolerance, threatening their health, well-being, and survival. The disturbance of homeostasis is known as stress and is triggered by stressors, which are agents that induce a coordinated set of responses involving the alteration of physiological regulation. Stressors can be physical, chemical, biological, and/or social (Barton et al., 2002). Among physical stressors, routine management procedures during rearing can be highlighted; among chemical stressors are changes in the aquatic environment resulting from rearing or from anthropogenic actions; and biological/social stressors include stocking density, the presence of predators, the establishment of social hierarchy, and interaction with macro or microorganisms.

In stressful conditions, the fish present responses that can be grouped into three categories: those that start immediately after the detection of the stressor at the neuroendocrine level, which are called primary responses (perception of the stressor, alarm for the mobilization of biological systems); those resulting from neuroendocrine action, which involve many organs and tissues and are called secondary responses (adjustments of the body for adaptation and resistance); and, lastly, responses characterized by the loss of adaptive capacity and exhaustion of biological systems, which are called tertiary responses. This chapter will highlight the mechanisms involved in the failure of the immune system's that increases the chances of an infection may be enhanced (Tort, 2011).

Organization and mechanisms of the stress responses

Primary stress responses: Alarm for the mobilization of biological systems of adaptation and resistance

The primary stress response in fish begins with the recognition of a stressor and the installation of a neural alarm mechanism involving two distinct axes: brain-sympathetic-chromaffin tissue and hypothalamus-pituitary-interrenal tissue (HPI) (Wendelaar Bonga, 1997, 2011) (Fig. 5.1). The activation of the stress system occurs seconds after the stressor through the activation of the sympathetic division of the autonomic nervous system in the hypothalamus and the subsequent release of catecholamines, adrenaline, and noradrenaline, and, in a lower amount, dopamine by chromaffin cells located on the walls of the posterior cardinal vein in the kidney cephalic portion. Chromaffin cells in teleosts are located close to steroidogenic interrenal cells and lymphoid tissue and are innervated by preganglionic cholinergic sympathetic fibers as well as by

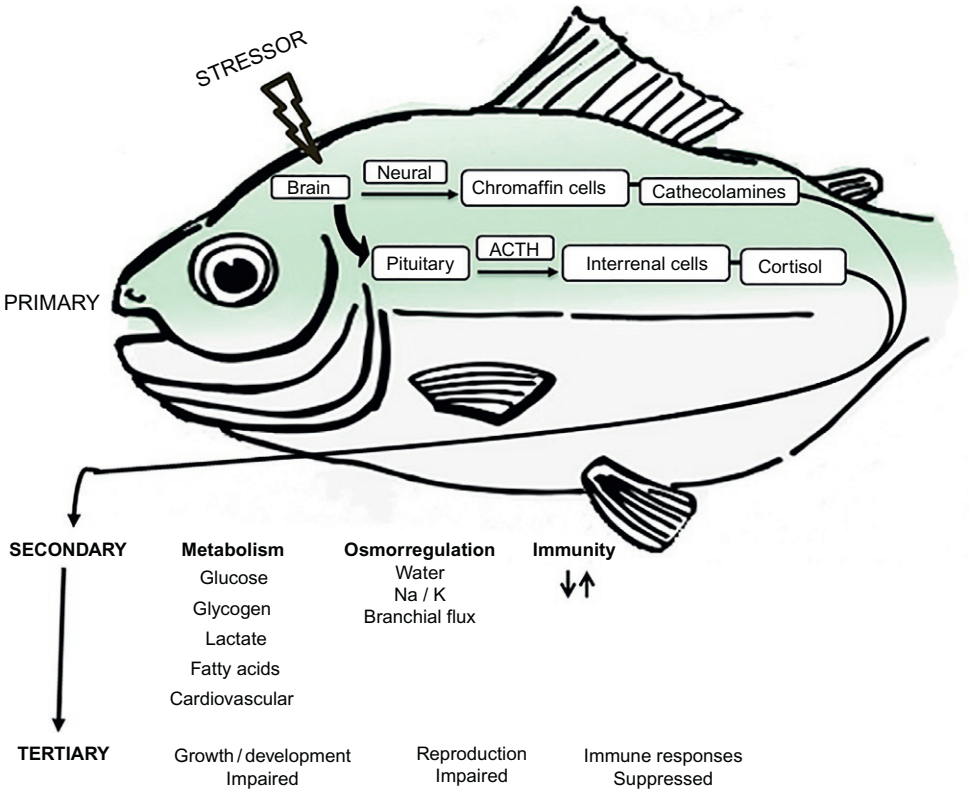

FIG. 5.1 Activation of the brain-sympathetic-chromaffin tissue and hypothalamus-pituitary-interrenal tissue axes and the set of the stress responses (primary, secondary, and tertiary). *(Adapted from Schreck, C. B., Tort, L., 2016. 1—The concept of stress in fish. In C.B. Schreck, L. Tort, A.P. Farrell, C.J. Brauner (Eds.), Fish Physiology. Academic Press, pp. 1–34).*

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noncholinergic fibers, with serotonin, ACTH (adenocorticotropic hormone or corticotropin) angiotensin II, histamine, VIP (vasoactive intestinal polypeptide), PACAP (pituitary adenylate cyclase-activating polypeptide), nitric oxide (NO), and hydrogen sulfide (H_2S) as catecholamine secretagogues, which ensure flexibility in the secretion process in response to stressors (Perry and Capaldo, 2011). The close location of the chromaffin and interrenal tissue suggests a paracrine interaction between catecholamines and cortisol. High cortisol concentrations increase the concentrations of catecholamines stored in chromaffin cells in rainbow trout (*Oncorhynchus mykiss*) as well as the sensitivity of these cells to cholinergic stimulation (Reid et al., 1996).

Following the activation of the brain-sympathetic-chromaffin cell axis by the stressor, and after longer latency (minutes), the HPI axis is activated in a cascade of reactions. The central nervous system, through higher neural centers, stimulates neurons from the preoptic nucleus of the hypothalamus to produce the corticotropin-releasing hormone (CRH). These neurons project to the pituitary gland and stimulate the release of corticotropin (ACTH) by cells from the anterior portion of the gland. CRH neurons also produce the arginine-vasotocin peptide, which potentiates the stimulation of ACTH cells by CRH. ACTH, when released into the blood, stimulates the interrenal cells to produce glucocorticoids, especially cortisol, that, together with catecholamines, act to organize the secondary adaptive responses in organs and tissues (Wendelaar Bonga, 1997, 2011).

In addition to ACTH, other pituitary mediators are activated during the primary stress response. Evidence shows that thyroid hormones (TH), thyroxine (T_4), and triiodothyronine (T_3) can modify the profile and intensity of stress responses in fish as well as modify their own actions and the actions of the interrenal stress hormones (Peter, 2011). According to this evidence, the hypothalamic and pituitary hormones of the thyroid and interrenal axes can interact with each other and regulate the actions mediated by TH and cortisol. Although it is not yet known how this interaction occurs, the intensity of the stress response in fish depends on the conditions of the THs, indicating the functional relationship with the interrenal axis. Evidence also suggests that CRH activates melanocyte-stimulating hormone (α-MSH) and *N*-acetyl-β-endorphin (NAc β-end) producing cells in common carp (*Cyprinus carpio*), and that stimulation may vary depending on the responsiveness of the cell and the intracellular signaling mechanisms (van den Burg et al., 2005). Several hypothalamic factors are involved in regulating the secretion of corticotropic, melanotropic, and thyrotropic cells in fish. Among these factors, CRH and TRH stimulate while dopamine, in general, inhibits the secretions of the three hypothalamic axes. Multiple interactions have been identified between these endocrine axes, forming a "stress web" that acts with highly coordinated actions on the energy metabolism as the main focus (Bernier et al., 2009). Recently, Skrzynska et al. (2018) demonstrated in gilthead sea bream (*Sparus aurata*), both at the transcriptional and circulating levels of several hormones, the existence of a complex activation of different endocrine pathways related to the stress pathways where vasotocinergic and isotocinergic systems can also be considered key players of the acute stress response orchestration. The authors induced a stress condition by air exposure for 3 min and investigated the transcriptomic response of different endocrine factors (CRH, POMC—proopiomelanocortin, TRH), neuropeptides (arginine vasotocin, Avt, and isotocin, It), and their specific receptors in the hypothalamus, pituitary, kidney, and liver. They also investigated circulating levels of catecholamines and cortisol. The plasma levels of noradrenaline, adrenaline, and cortisol increased a few minutes after stress exposure. At the hypothalamic and hypophyseal levels, acute stress affected mRNA expression of all measured precursors and hormonal factors as well as their receptors (avtrs and itr), showing activation at the central level of the hypothalamus-pituitary-interrenal (HPI) and the hypothalamus-sympathetic-chromaffin cell (HSC), and Avt/It axes in the acute stress response. In addition, stress response also affected mRNA levels of their receptors in the head kidney as well as the steroidogenic acute regulatory protein (star) and tyrosine hydroxylase (th) expression, suggesting their participation in the HPI and HSC axes activation. Moreover, the pattern of changes in hepatic gene expression of both receptors also highlights an important role of vasotocinergic and isotocinergic pathways in liver metabolic organization after acute stress events. Another pituitary peptide that has been associated with the stress response is somatolactin (SL), which is produced by cells of the pars intermedia of the fish pituitary and related to gonadal maturation, smolting, skin pigmentation, lipid metabolism and energy mobilization, acid-base regulation, immune function, and calcium, sodium, and phosphorus regulation. However, their levels were markedly elevated during stress caused by confinement in chinook salmon (*Oncorhynchus tshawytscha*) as well as during different periods of handling and confinement in rainbow trout (Randweaver et al., 1993). The SL increase occurred 2 min after the stressor, preceding the increase in circulating levels of cortisol.

Secondary stress responses: Adaptive response to the maintenance of organic homeostasis

The hormones released within seconds to minutes after exposure to the stressors trigger metabolic and osmoregulatory changes to meet the energy demand of the animal (Fig. 5.1). The increase in circulating glucose levels is initially and in part due to the catecholamine action on the hepatic glycogen breakdown (Mazeaud and Mazeaud, 1981). Glucose is

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the primary energy source for fish and is used primarily to meet brain and muscle needs "to fight or flight." Catecholamines regulate cardiac and respiratory functions, including increased branchial blood flow, branchial oxygen diffusing capacity, and the increased oxygen transport capacity of the blood (Wendelaar Bonga, 2011). In freshwater fish, this leads to the loss of blood ions with reduced blood concentrations of sodium and chloride. Catecholamines are also responsible for the increase in the number of red cells due to splenic contraction as well as a higher affinity of hemoglobin. These actions optimize cardiorespiratory function and ensure an adequate supply of oxygen to tissues (Reid et al., 1998). Along with catecholamines, cortisol is responsible for the physiological preparation of the fish to cope with the stressor, inducing metabolic, osmoregulatory, and immunological responses. Cortisol is necessary to maintain high blood glucose levels and to recover hepatic glycogen levels. It can act initially in the glycogenolytic process, along with catecholamines, mainly to increase the gluconeogenic capacity when there is a mobilization of amino acids from body proteins. Lactate can also be used as a gluconeogenic source in situations of higher availability (Mommsen et al., 1999). Regarding osmorregulatory responses, evidence shows that cortisol is an important hormone for the acclimatization of fish in freshwater, having both glucocorticoid and mineralocorticoid actions. It acts on the function of the gill cells, on the proliferation and differentiation of the gill ionocytes (chloride cells), and on the reabsorption of sodium in freshwater fish. The mineralocorticoid functions of cortisol occur by the stimulation of Na-K ATPase activity in the gills, intestines, and kidneys (Wendelaar Bonga, 1997, 2011).

In addition to the physiological and biochemical stress responses, stress conditions and metabolic insults can also act directly at the cellular level and promote molecular changes (Yamashita et al., 2010). Heat shock proteins (HSPs) are a family of highly conserved cellular proteins that have been observed in all organisms, including fish (Feder and Hofmann, 1999; Iwama et al., 1999). Three major families of HSPs [HSP90 (85–90·kDa), HSP70 (68–73·kDa), and low molecular-mass HSPs (16–24·kDa)] have been studied. They are required in various aspects of protein metabolism and play a central role in cellular homeostasis (Fink and Goto, 1998). Cell stress responses, which are multiple and stressor-specific, include stimulated or induced expression of proteins, such as the HSPs in various fish tissues (Iwama et al., 2004). It was suggested that cortisol may mediate HSP70 levels in fish tissues following physiological stress and that neuroendocrine and cellular stress responses may be functionally related (Basu et al., 2001). Souza-Bastos et al. (2016) compared the physiological effects of exposure to increased salinity in the neotropical fish pacu (*Piaractus mesopotamicus*) and tambaqui (*Colossoma macropomum*) and in their hybrid (tambacu) and found that salinity exposure resulted in no change in HSP70 expression as quantified through Western Blotting, for both the species and their hybrid.

Tertiary stress responses: Exhaustion of biological systems

If a stressful condition extends, secondary stress responses change their characteristics and tertiary responses settle (Fig. 5.1). During the adaptive phase, energy is diverted to priority organs and functions involved in survival such as breathing, swimming, osmoregulation, and tissue repair, reducing the contribution to long-term anabolic activities such as growth, reproductive process, and immune functions. If the stressor is of an acute nature, homeostasis can be recovered without severe consequences to fish; however, stressors of a chronic nature lead the organism to compromise its energy stores, with the installation of physiological exhaustion, a reduced rate of growth and reproductive capacity, and the severe impairment of immune functions (Barton et al., 2002; Wendelaar Bonga, 1997). The impairment of the fish's defense system affects its health, well-being, and survival, with diseases commonly appearing (Walters and Plumb, 1980).

Fish immune system

The innate and acquired immune system of teleost fish

The immune system of the fish is a set of cellular and humoral components whose function is the organism's defense against foreign substances, such as microorganisms, toxins, or malignant cells (Bols et al., 2001; Magnadottir, 2006; Rauta et al., 2012; Secombes and Wang, 2012). It is divided into innate or nonspecific and adaptive/acquired or specific, both of which present cell-mediated defense mechanisms and humoral factors. These two divisions of the immune system work together to destroy invaders or trigger defensive processes. The innate system is the first line of defense against invading agents, whereas acquired immunity is related to the elimination of the pathogen in a late stage and the production of immunological memory (Ye et al., 2018).

The components of the innate system are the tegument (skin and mucus); the cellular components, which are defense cells (granulocytes, monocytes, macrophages, and natural killer cells); and humoral components (the complement system, antimicrobial enzyme system, and nonspecific mediators such as interferon and interleukins) (Ellis, 1999). The surface of the fish's body presents a mechanical barrier to microorganisms and, in aquatic organisms, the outer mucus and internal

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epithelial surfaces contain antimicrobial compounds that include lectins, complement, lysozyme, and bactericidal peptides. The integument consists of mucus and skin, an epithelial barrier with specialized cells secreting bactericidal components that prevent the entry of harmful microorganisms. The alimentary canal also acts as a barrier against the entrance of microorganisms because its content of acids, bile salts, and enzymes creates a hostile environment for noncommensal microorganisms. In most cases, tegumentary barriers are sufficient to block the invading organism; however, if it penetrates tissues and circulation, it will be recognized by cellular and humoral components of the immune system (Ellis, 1999). The existence of effective mechanisms to prevent the entry and installation of microorganisms increases the possibility of the fish's survival (Bayne and Gerwick, 2001; Ellis, 2001).

Inflammation is also considered an innate mechanism of immune response mediated by complex interactions of cellular and humoral compounds. After tissue invasion by an infectious agent, mediators are released, promoting vasodilation and increasing the permeability of blood capillaries to facilitate the migration of defense cells. Granulocytes are the first cells to reach the focus of inflammation and are responsible for the destruction of pathogens. Cell debris and remaining pathogenic cells are later phagocytosed by macrophages that replace granulocytes (Magnadottir, 2006).

The specific defense system activation depends on the presence of the antigen that triggers the cascade of reactions responsible for the increase of circulating antibodies specific for the invaders, in addition to promoting immune memory (Bernstein et al., 1998). After penetrating the organism, antigens are recognized and captured by the antigen-presenting cells (APCs—macrophages, dendritic cells, and B lymphocytes). These cells process the microorganisms in molecular units, and at first, they trigger the immune and proliferation response, then the memory response (Abbas and Lichtman, 2004). In this pathway, the antigen will be presented by the APCs to the T lymphocyte, a specific system cell that has the ability to recognize the antigen strictly in the presence of specific humoral components called histocompatibility molecules, glycoprotein receptors encoded by genes in a complex called the major histocompatibility (MHC). After recognition, the T cell secretes cytokines, which are proteins that activate B lymphocytes (responsible for antibody production), cytotoxic lymphocytes, macrophages, and other cells that act to destroy the invading agent (Goldsby et al., 2000). Antibodies bind to microorganisms and activate phagocytosis, promoting the neutralization and opsonization of the agent as well as complementing the activation and antibody-dependent cell-mediated cytotoxicity (Ellis, 2001). Antibodies have the ability to bind to extracellular antigens in the mucosa and bloodstream. If they are lodged in the intracellular compartment, defense is performed by cytotoxic T lymphocytes (Goldsby et al., 2000). The receptors in the acquired system responsible for detecting the invading agent are found on the membranes of immunocompetent cells, T lymphocytes (TCR), and B lymphocytes (BCR, surface immunoglobulin) (Abbas and Lichtman, 2004).

Cell-mediated immune system and humoral compounds

Cell-mediated immune system

The defense cells of the teleost fish are produced by lymphoid tissues such as the kidney, thymus, spleen, and mucosa-associated tissues by hematopoiesis. During hematopoiesis, cell formation and maturation and the differentiation of erythrocytes, granulocytes, monocytes, mast cells, lymphocytes, and thrombocytes can occur, depending on the stimulus (Evans et al., 1997). Hematopoiesis in fish occurs in the kidneys, which produce B cells, monocytes, macrophages, and granulocytes; in the thymus, which produces and matures the T lymphocytes; and in the spleen, which produces lymphocytes and macrophages. In addition, the mucosa-associated lymphoid tissues produce macrophages, lymphocytes, mast cells, and granulocytes (Georgopoulou and Vernier, 1986). Hematopoiesis is regulated by cytokines that act on the receptors of the lymphoid pluripotent cell tissue, controlling their survival, proliferation, differentiation, maturation, and function (Hanington et al., 2009).

Among the cells produced during hematopoiesis, thrombocytes are blood cells with the function of hemostasis, homeostasis, and phagocytosis; they usually have an inflammatory focus (Tavares-Dias et al., 1999). Another important cell for innate defense is the neutrophil, a granulocyte that contains antimicrobial and cytotoxic substances and respiratory burst activity that acts on intracellular and extracellular pathogens (Rieger and Barreda, 2011). Monocytes are nonspecific phagocytes with cytotoxic activity in blood; however, when they migrate to tissues, they are called macrophages and present a great capacity of respiratory burst activity (Cuesta et al., 1999; Rieger and Barreda, 2011). Eosinophils act in the process of inflammation and cellular defense by degranulation and are distributed in the connective tissue, especially in the gills, gastrointestinal tract, and bloodstream when there is an infestation of parasites. Basophils are rare in most fish (Hine, 1992). Special granulocytic cells or PAS-positive granular leukocytes are polymorphonuclear cells, and their exact function is not yet known; they occur in a greater amount in fish parasitized or injected with phlogogenic agents (Martins et al., 2000).

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The T and B lymphocytes are the cells of the adaptive system. However, there is a type of lymphocyte that does not have the same characteristics as T or B and forms a distinct lymphocyte population, known as natural killer or NK cells, that lyse foreign cells or cells infected by a virus, in addition to producing immunoregulatory cytokines (Raulet, 2004). Monocytes, macrophages, granulocytes, and dendritic cells are professional phagocytes; however, other cells can phagocyte. For this, recognition of the invading agent by membrane receptors is required. At the entry sites of the agents, molecular signals of inflammation (cytokines) are released by the damaged tissue and cells promote chemotaxis and the mobilization of phagocytes to the site of invasion (Stuart and Ezekowitz, 2005). Neutrophils and macrophages have great importance in the organism's defense as they phagocyte and destroy the microorganisms, mainly due to the action of the reactive oxygen species (ROS) produced during the respiratory activity (oxidative burst) of the cells, an oxidative and hydrolytic enzymatic reaction stimulated by the microorganisms (Secombes et al., 1996). During phagocytosis, the consumption of molecular oxygen increases due to the reduction of oxygen in the superoxide anion, which, by the action of the superoxide dismutase (SOD), forms hydrogen peroxide (H_2O_2). H_2O_2 is a substrate for the myeloperoxidase enzyme (MPO) released by the granular leukocytes, and as a result, there is the formation of hypochlorite with the consequent production of chloramine. The reactive radicals produced are oxidizing agents that act on the membranes of microorganisms and contribute to their destruction (Verlhac et al., 1998). During the inflammatory response, cells tend to migrate due to chemotactic factors released in the inflammatory focus. Circulating neutrophils are the first granulocytes to reach the lesion site, followed by then the monocytes/macrophages derived from the blood monocytes. At the site of injury, these cells initiate the phagocytosis process for destruction of the invading agents (Rowley, 1996).

The macrophage-lineage cells are the cellular populations that kill pathogens through phagocytosis and the production of reactive oxygen and nitrogen molecules, inflammatory cytokines, chemokines, and lipid mediators (Grayfer et al., 2018) as well as by triggering polarized immune responses (Wiegertjes et al., 2016). The defense cells of the innate system also have the function of promoting the connection with the acquired system. This binding is performed by antigen-presenting cells (dendritic cells and macrophages) that, upon processing, present the invader to T lymphocytes with the collaboration of the major histocompatibility complex (MHC) class-2 molecules, initiating the cell-mediated acquired response.

Humoral immune system

The humoral innate immune system acts through soluble components of bodily fluids (Bayne and Gerwick, 2001). The mechanisms that defend against the invasion of pathogens include the production of antibacterial compounds, the acute phase proteins of inflammation, the complement action activated by the alternative pathway, cytokines, phagocytosis, and inflammation (Ellis, 1999, 2001). Innate humoral factors include inhibitory factors for the growth of bacteria, such as transferrin, antiproteases, lysozyme, C-reactive protein, antibacterial peptides, and complement system proteins activated by the alternative pathway and lectins; the latter is the most important because they have lytic, proinflammatory, chemotactic, and opsonizing activity, influencing the action of defense cells. Among defense cells, neutrophils and macrophages are especially important because they contain large amounts of lysosomal enzymes. Transferrin, a soluble protein present in the blood, is considered an activator protein of macrophages and a conjugating protein of metals, with high affinity for iron ions. It is produced mainly in the acute phase of inflammation (Bayne and Gerwick, 2001), and its function is to bind to the iron ions, which are essential for bacterial proliferation and consequently the establishment of infection. Transferrin makes iron unavailable, interfering with the infection (Bullen and Griffiths, 1987). Antiproteases are present in blood and act on bacteria proteolytic proteins that digest host tissue to obtain amino acids as a nutritional source, avoiding the use of energy for bacteria multiplication. Lectins are proteins found in eggs, mucus, and blood that have a high affinity for carbohydrates that are part of the cell wall of bacteria, promoting the agglutination of pathogens (Arason, 1996). Another important group of humoral factors is the proteases present mainly in mucus (Braun et al., 1990). They are antibacterial peptides that attack the membranes of pathogens (Ellis, 1999, 2001). C-reactive protein is a pentraxin family protein found in large concentrations in fish blood, eggs, and mucus. It binds to phosphorylcholine on the walls of microorganisms such as bacteria, fungi, and parasites (Yano, 1996). C-reactive protein and mannose-binding lectin (MBL) are considered acute phase proteins and soluble receptors of microbial components. They bind and promote their opsonization (Goldsby et al., 2000) and also activate the complement system and phagocytosis (Nakanishi, 1991). Lysozyme is another important nonspecific component of the immune system found in several species of fish (Grinde et al., 1988). This bactericidal enzyme has lytic activity against Gram-positive and Gram-negative bacteria, parasites, and viruses (Magnadottir, 2006). It is also found in mucus (Palaksha et al., 2008), lymphoid tissues, plasma, and other fluids in the body where leukocytes exist, mainly monocytes and neutrophils (Murray and Fletcher, 1976). The complement system represents the main effector of the innate immune response and one of the most important components in the defense against invading pathogens (Koppenheffer, 1987); it is also considered one of the main mediators of the inflammatory process, attracting

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phagocytic cells to the lesion site (Nonaka et al., 1981). The complement system is a set of about 35 soluble and membrane proteins that act in a cascade of enzymatic reactions to perform phagocytosis, opsonization, chemotaxis of leukocytes, and inactivation of toxins released by bacteria (Secombes et al., 1996) through the activation of three different pathways: (i) the classical pathway, which is antibody-dependent through an antigen-antibody complex activation; (ii) the alternative pathway, which is activated by surface molecules of microorganisms and by the antigen-antibody complex; and (iii) the lectin pathway, which is activated by the binding of bacterial surface carbohydrates. These three pathways have already been identified in fish (Holland and Lambris, 2002). The action of classical, alternative, and lectin pathways forms the membrane attack complex, which is responsible for lytic activity in pathogens (Nakao et al., 2011). The alternative pathway is considered the most important for fish because of its high efficiency in innate defense mechanisms. This pathway can be activated by membrane lipopolysaccharides of various Gram-negative bacteria, and can cause cytolysis. During complement activation, two components, C5a and C3b, are very important for phagocyte recruitment. C5a is a chemotactic protein for neutrophils and macrophages, which have receptors for C3b, remaining bound to the bacterial wall and facilitating phagocytosis (Ellis, 2001; Yano, 1996).

The innate humoral components activated after the invasion of pathogens (bacteria, viruses, parasites, and fungi), traumas, necroses, irritating chemicals, burns, and tumor cells are called acute phase proteins and the body's response is called the acute phase response. The vast majority of these components are synthesized by the liver, but also in the brain and leukocytes (Bayne and Gerwick, 2001).

Stress, immune, and inflammatory responses: Changes in immunocompetence during stress response

Acute responses during the initial phase of the stress response appear to increase innate immunity, whereas more prolonged stress conditions reduce immunocompetence (Dhabhar, 2000). Activation and suppression of the innate response have already been described in fish and are related to the type of stressor and times of exposure. A study with Atlantic salmon (*Salmo salar*) evaluated the effects of acute stress (aerial exposure for 15 s) and chronic stress (aerial exposure for 15 s daily for four weeks) on stress indicators (blood cortisol and glucose) and the innate immunity (expression of the inflammatory gene interleukin *il1β*, survival of cephalic kidney macrophages in culture with *Aeromonas salmonicida*). The results showed that chronic stress may have imposed an overload on fish that compromised their immune function. Acute stress resulted in increased glucose and cortisol concentrations and high expression of the *il1β* gene. In the chronic condition, the stress indicators did not change but there was an inhibitory effect on the immune response, with reduced stimulation of the macrophages by LPS and reduced survival in the presence of bacteria (Fast et al., 2008). Increased lysozyme levels were observed after acute stress in rainbow trout (Demers and Bayne, 1997) while sea bass (*Dicentrarchus labrax*) confined at high densities showed immunosuppression with reduced phagocytic activity, as shown by the production of reactive species of oxygen by cephalic kidney phagocytes and reduction of the cytotoxic activity of the peritoneal leukocytes. The stress indicators were correlated with cellular immunity by linear regression (Vazzana et al., 2002).

Immune system and modulation by catecholamines

The activation phase of the immune system is related to the rapid production of acute phase proteins and the release of cytokines, hormones, and peptides. This response is partly mediated by the catecholamines released during the primary stress response, as discussed before. Blood cells, including erythrocytes and leukocytes, are mobilized as part of the acute stress response by the activation of the autonomic nervous system and the release of catecholamines (Tort, 2011). However, studies involving the role of the sympathetic nervous system in mediating the effect of stress on immune responses of fish are still limited, and the results are contradictory. The effect of acute and chronic stress on macrophage phagocytic activity was evaluated in rainbow trout. It was proposed that the autonomic nervous system could be a regulator of phagocytosis at the earliest stage of stress, depressing phagocytic activity, and that corticosteroids would only play a role in long-term suppression (Narnaware et al., 1994). Recently, Zanuzzo et al. (2019) compared how stress and corticosteroids modulate the innate immune response in pacu, using two strategies: dietary corticosteroids such as dexamethasone or hydrocortisone (seven days; long-term exposure), and/or transport (4 h; short-term stress). Both corticosteroids reduced the hemolytic activity of the complement while they increased serum lysozyme concentrations. The transport increased cortisol and reduced the humoral immune defenses such as serum lysozyme concentration and hemolytic activity of the complement system (alternative pathway). Interestingly, the hemolytic activity of the complement system increased sharply in fish fed with corticosteroids immediately posttransport, when they had their HPI axis partially suppressed by the corticosteroids, which suggests a stimulatory effect of the catecholamines released during the transport on the activity of the complement

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system. Additionally, the serum lysozyme concentration increased immediately after chasing and air exposure in pacu, without changes in cortisol levels (Soares et al., 2018).

To understand the role of the sympathetic nervous system on interrenal tissue regarding the stress effect on innate immune responses in fish, Roy and Rai (2008) through *in vivo* and *in vitro* trials studied the phagocytic activity and respiratory activity of macrophages in normal and chemically sympathectomized *Channa punctatus* under restricted conditions. The sympathectomy abolished the differential effects of acute stress on various macrophage functions while the *in vitro* trials indicated the action of catecholamines by different receptors and intracellular pathways, justifying the controversial responses of macrophages. In accordance with Roy and Rai (2008), an *in vitro* study using agonists and β-adrenergic receptor antagonists showed inhibitory effects on innate and acquired immune system responses while α-adrenergic receptor agonists caused stimulation of the respiratory activity of leukocytes and antibody production in various fish species (Verburg-Van Kemenade et al., 2009). Recently, types of adrenergic receptors have been described in fish (Verburg-Van Kemenade et al., 2011), and several studies have shown that acute stress can stimulate immune responses (Dhabhar, 2000, 2009; Dhabhar and McEwen, 1997), which, consequently, is related to the release of catecholamines (Chadzinska et al., 2012).

Immune system and modulation by cortisol

One of the main modulators of the neuroimmunoendocrine system is cortisol. The immunosuppressive effects of stress are mainly associated with the action of corticosteroids of the hypothalamic-pituitary-interrenal axis. Evidence indicates that high levels of circulating cortisol are associated with immunosuppression in fish, as occurred in seabream (*Sparus aurata*) after thermal shock (Tort et al., 1998) or confinement at high densities (Montero et al., 1999). Stress caused by thermal shock in common carp affected the leukocyte population, which decreased after changes in temperature (Engelsma et al., 2003). A high level of cortisol was found to affect cytokine production by immune system cells (Verburg-Van Kemenade et al., 2009), induce apoptosis in B cells (Weyts et al., 1998a) and have suppressive effects on various immune responses in fish, including phagocytosis, leukocyte respiratory activity (Esteban et al., 2004), and lymphocyte mitogenesis (Harris and Bird, 2000). They also decreased the activity of circulating antibodies and circulating IgM-producing cells (Nagae et al., 1994), and in general caused a rapid increase in neutrophils (Weyts et al., 1998b) and a reduction in lymphocytes, probably due to distribution in the gills, skin, and intestines (Wendelaar Bonga, 1997). Recently, Zanuzzo et al. (2017) showed that 4 h transport increased the blood cortisol level and impaired both the leukocyte respiratory burst and the hemolytic activity of the complement system in the neotropical fish pacu. The cortisol inhibitory effect on the immune system althought jeopardize the immune system in terms, could be also important because the production of microbicidal proteins, inflammatory cytokines, reactive oxygen species, and a large number of cytotoxic cells in the circulation can also be potentially harmful and even lethal to the host. Thus, understanding this complex bidirectional neuroimmunoendocrine system is necessary to establish strategies for the prevention and treatment of infectious diseases in fish (Verburg-Van Kemenade et al., 2009).

In fish, two types of glucocorticoid receptors (GRs), GR1 and GR2, are known (Stolte et al., 2006), and GR1 has two variants: GR1a and GR1b, so that four receptors are capable of binding to cortisol in fish: GR1a, GR1b, GR2, and MR (mineralocorticoid receptors) (Verburg-Van Kemenade et al., 2009). However, the ability of cortisol to activate genes depends on its circulating concentration. In rainbow trout and common carp (*Cyprinus carpio*), GR2 can be activated at low cortisol concentrations, whereas the GR1 receptor is sensitive to high levels of the hormone (Stolte et al., 2008). Because of its lipophilic nature, cortisol passes freely through the cell membrane and acts at the gene level by binding to nuclear glucocorticoid receptors (GR) (Pruett, 2003). This binding causes a dissociation of HSP90 and other receptor proteins that allows the migration and signaling of glucocorticoids to the nucleus (Almawi and Melemedjian, 2002). The promoter region in the nucleus has numerous glucocorticoid response element genes (GREs), and the binding of receptors to such binding sites may induce or suppress gene transcription (Aluru and Vijayan, 2009; Weyts et al., 1998b). Delayed glucocorticoid effects are mediated by mechanisms involving transcriptional regulation; however, relatively rapid effects of glucocorticoids also occur and invoke a noncanonical mode of steroid action. Studies conducted on different species suggest that the rapid effects of glucocorticoids (GCs) are mediated by the activation of one or more membrane-associated receptors (Tasker et al., 2006). Experimental evidence has accumulated that GCs can also act rapidly through a nongenomic mechanism to modulate cellular physiology in vertebrates. Evidence suggests that cortisol exerts rapid, nongenomic actions in the gills, liver, and pituitary in the Mozambique tilapia (*Oreochromis mossambicus*), but the membrane receptor mediating these actions has not been characterized (Johnstone 3rd et al., 2013). A recent review suggests that cortisol activates multiple signaling pathways in cells to bring about rapid effects, but the underlying physiological implications on acute stress adaptation are far from clear (Das et al., 2018).

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Modulation of the immune system by immunostimulants

Other substances that modulate the immune system and improve the immunocompetence of fish are immunostimulants, which may promote the activation of specific or nonspecific mechanisms (Saurabh and Sahoo, 2008). Studies with immunostimulants have brought advances to the understanding of the innate immune system in fish, besides being an alternative method in the prevention of common diseases in the fish culture (Galina et al., 2009). The use of antibiotics has encountered resistance worldwide due to the consequences related to increased resistance developed by pathogens as well as the toxicity and environmental impact (Lim et al., 2013; Sanderson et al., 2004). Immunostimulants may act synergistically with antibiotics, and their effects may be enhanced by nutritional factors (vitamin C and selenium), but no nutritional factor can be defined as an immunostimulant alone (Raa, 1996).

The best known immunostimulants are lipopolysaccharides (LPS) (Goetz et al., 2004), peptideoglycans (Zhou et al., 2006) and glucans (Dalmo and Bogwald, 2008), synthetic compounds (levamisole; FK-565), polysaccharides (chitin, chitosan), hormones, vitamins, and plant extracts that may increase the nonspecific immune responses of fish (Raa, 1996; Sakai, 1999). Immunostimulants, such as β-glucan (Chettri et al., 2013; Rodriguez et al., 2009), lactoferrin (Kumari et al., 2003), and levamisole (Maqsood et al., 2009) lead to the activation of innate and acquired immune mechanisms (Anderson and Jeney, 1992; Ganguly et al., 2010) and have been shown to be suitable for use in aquaculture (Sakai, 1999), improving immune responses and resistance to diseases (Bricknell and Dalmo, 2005). In addition, the use of medicinal plants in aquaculture (Dugenci et al., 2003; Galina et al., 2009) has also been shown to be an alternative option (Citarasu, 2010). Several studies have shown the efficiency of some plants in the stimulation of immune responses and protection against bacterial infestation in several species of fish (Yin et al., 2009; Zanuzzo et al., 2012; Zanuzzo et al., 2017).

Recently, mechanisms of trained immunity have been observed following the administration of some immunostimulants, such as glucan, that may exhibit the ability to achieve early immunity, avoid mortality, and increase survival (Petit and Wiegertjes, 2016; Rojo-Cebreros et al., 2018). Trained immunity, or the prolonged innate defense, is characterized by an innate immune system activation in which the recognition of pathogens, in addition to promoting a specific defense, promotes a prolonged innate defense after a second infection with the same pathogen; this can also establish cross-protection, possibly promoted through epigenetic mechanisms (Netea et al., 2016; Rojo-Cebreros et al., 2018).

A review on propolis, a bee honey product, has shown its immunomodulatory and antitumor properties, taking into account effects on the production of antibodies and immune cells that involve innate and acquired responses. Its inhibitory effects on lymphoproliferation may be associated with its antiinflammatory properties (Sforcin, 2007). Propolis has also been used in fish (Cuesta et al., 2005). Another natural product used as an immunostimulant is garlic (*Allium sativum*), which, along with its extracts, has been widely used to treat infections for thousands of years (Koch, 1996). The application of garlic in the treatments of various fish diseases and their potential use in aquaculture has recently been revised (Lee and Gao, 2012). Garlic's protective action against infection by *Aeromonas hydrophila* and its innate immune defense stimulating action, such as the increased production of oxidative radicals by neutrophils, the proliferation of lymphocytes, and the phagocytic activity of cephalic kidney macrophages, has been demonstrated in fish (Nya and Austin, 2009).

Mechanism of action of immunostimulants

The mechanism of action of some immunostimulants is related to toll-like receptors (TLRs), a class of proteins that plays a key role in the innate immune system (Takeda et al., 2003). Their mode of action is highly sophisticated and consists of a very specialized system that can identify several endogenous microorganisms and ligands as well as activate the ideal immune response for each antigen (Heine and Lien, 2003). In fish, at least 11 types of receptors have been discovered, each with different functions and ligands (Takano et al., 2010). In addition, the TLR-2, TLR-5, and TLR-9 receptors were highly expressed in *Danio rerio* (Meijer et al., 2004). In Japanese flounder (*Paralichthys olivaceus*), 11 types of TLR homologues were identified (Hwang et al., 2011). Studies of TLRs have opened a new area of research with great potential in the field of fish immunology, benefiting from the growing use of innovative technologies for knowledge and evaluation of the immune system, such as transcriptomics and proteomics analyses to evaluate the modulation of the immune system (Ye et al., 2018).

Modulation of the immune system by cytokines

As discussed above, the immune system is controlled by a complex bidirectional neuroendocrineimmune system mediated by hormones and cytokines (Engelsma et al., 2002; Verburg-Van Kemenade et al., 2011; Verburg-Van Kemenade et al.,

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2009). Although little is known about the cytokines in neotropical freshwater fish, many aspects of the regulation of immune responses by cytokines are relatively well conserved in teleost (Zou and Secombes, 2016). Thus, in this section, we briefly discuss the most important cytokines and their importance in modulating and coordinating immune responses in an overall view for fish.

Cytokines are structurally classified as glycoproteins or polypeptides of low molecular weight (less than 30 kDa). They work as an "informant," connecting and isolating several components of the immune system in order to mount and drive immune responses. Cytokines may act in an autocrine or paracrine manner in various target cells responsible for the modulation of host defense (Savan and Sakai, 2006), and their effects may be pleiotropic (Secombes et al., 1996). They are usually produced at the site of entry of the pathogen by activated immune cells, but many cytokines may also be secreted by cells of the central or endocrine nervous system. For example, astrocytes and microglial (central nervous system) cells may produce tumor necrosis factor (TNF), interferons INF-α and INFγ-1, -2 and -6. In addition, hypothalamic and pituitary tissues may also produce transforming growth factor (TGF) and interleukins (IL-10 and IL-18)(Tort, 2011). Thus, some cells of the nervous system and endocrine system share the ability of the immune system to produce specific proteins that can act as immune modulators or metabolic regulators, which exemplify the neuroendocrineimmune interaction described above. Further, cytokines may also be categorized into different families according to their function, structure, and properties. They can be proinflammatory or antiinflammatory, correlated to bacterial and/or viral responses, and can also be involved in innate and/or adaptive immune responses.

The interleukins-1 (IL-1) family acts as a potent proinflammatory, and IL-1 is the most functionally described and characterized cytokine in jawed vertebrates (Bird et al., 2002; Huising et al., 2004; Savan and Sakai, 2006). IL-1 is secreted by a wide range of cells and was the first to be cloned in fish (Engelsma et al., 2003). Its effects include increased phagocytosis, lymphocyte proliferation, and the production of superoxides (Savan and Sakai, 2006). Another important family related to inflammation is the IL-6 family. They are highly pleiotropic cytokines that act both in innate and adaptive immunity. They induce the differentiation of B and T cells, inflammation, and hematopoiesis (Hirano, 1998). Tumor necrosis factors (TNFs) are also categorized as proinflammatory cytokines and have an essential role in apoptosis, cell proliferation, and the stimulation of immune responses (Savan and Sakai, 2006). TNF-α is one of the first cytokines released at an early stage during infection in fish (Zou and Secombes, 2016), and the TNF-α protein enhances phagocytosis activity (Grayfer et al., 2008), promotes macrophage survival, and restricts bacterial growth in infected macrophages (Saeij et al., 2003). Altogether, IL-1β, IL-6 and TNF-α are the main mediators of inflammatory responses in fish (Zou and Secombes, 2016). In response to viral infection, a number of cytokines are also released in order to coordinate the immune response, such as IFNs. They are classified into three groups (type I, II, or III) and have different structures, functions, and interactions with different receptors (Zhu et al., 2013). IFNs have been shown to upregulate and control a large number of genes during a viral infection such as Mx, viperin, ISG15, and IRF (Aggad et al., 2009; Altmann et al., 2003; Li et al., 2014; Poynter and DeWitte-Orr, 2016; Zou et al., 2007).

On the other hand, there are antiinflammatory cytokines that can suppress the immune response such as transforming growth factor-β (TGF-β) and IL-10. TGF-β may inhibit the nitric oxide response and the upregulation of TNF-α, IL-1β, and IL-8 (Haddad et al., 2008; Wei et al., 2015). The *il10* gene has been identified in a number of teleost fish (Grayfer et al., 2011; Piazzon et al., 2015), and it has been shown that IL-10 substantially decreases the expression of *tnfα1*, *tnfα2*, *il1β1*, *il8,* and *cxcl8* (Grayfer et al., 2011). In addition, IL-10 also inhibits the expression of genes involved in MHC (major histocompatibility complex) antigen presentation.

Supplementary to these cytokines, phagocytes may release substances called chemokines (Alejo and Tafalla, 2011), a specialized family of cytokines known as small cytokines, including IL-8, another potent inflammatory mediator (Harun et al., 2008; Montero et al., 2008). Their main function is to promote the recruitment and activation of neutrophils and monocytes to the focus of inflammation (Baggiolini et al., 1992; Gerszten et al., 1999) and to stimulate the respiratory activity of neutrophils and monocytes (Baldwin et al., 1991; Walz et al., 1991). Antimicrobial peptides (AMPs), such as cathelicidins and hepdicin, are another important category of immune-modulating components (Katzenback, 2015). In fish, these peptides appear to be an important multifunctional protein in defending against bacterial infection (Lu et al., 2011; Maier et al., 2008; Shewring et al., 2011). They act against bacteria by disrupting the cell membrane (Gennaro and Zanetti, 2000), having ample activity against pathogens, including Gram-positive and Gram-negative bacteria, fungi, and even some viruses (Bals and Wilson, 2003; Guglielmetti et al., 2009). In conclusion, cytokines have a wide range of functions, and they can modulate immune responses differently. The balance between proinflammatory and antiinflammatory cytokines is a trade-off that regulates the immune response to not be harmful for the fish, and in the meantime, be effective against pathogens.

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Strategies to improve the health of neotropical freshwater fish

The expansion of aquaculture has led to extra efforts in attempting to raise and intensify fish production, and this has been accompanied by an increase in disease outbreaks. Thus, the number of studies to seek out alternatives and develop new technology to enhance fish resistance to disease, such as strategies to reduce stress and/or strengthen immune defenses, has increased sharply. Among the strategies used to combat diseases, three methods have been widely adopted: vaccination, chemotherapy (antibiotic), and the use of immunomodulators (Anderson and Jeney, 1992; Sahoo, 2007). However, the vaccination of neotropical fish is still poorly developed for most species, and the majority of the vaccines available are effective against only one type of pathogen (Chandran et al., 2002) and usually are species-specific. Further, the development of vaccines for heterogeneous species or multiple strains is extremely complex (Dixon, 2012; Harikrishnan et al., 2011), and the vaccination is also stressful for juveniles (Murray et al., 2003). On the other hand, the use of antibiotics has led to several concerns regarding drug resistance, toxicity, and the environment, and their use has been drastically reduced (Lim et al., 2013; Sanderson et al., 2004). Thus, the use of immunomodulators appears to be a viable alternative for the prevention of diseases in fish. These compounds enhance the innate immune response, which acts broadly against pathogens, but they are not species-specific (Sakai, 1999). Although studies related to the strengthening of the immune system in neotropical freshwater fish are still scarce, the number is growing, and some research groups have demonstrated advances and several benefits of using immunomodulators such as vitamins, probiotics, prebiotics, and medicinal plants. Below, we review some of the main strategies used for neotropical freshwater species.

The use of micronutrients as an immunomodulator

A variety of micronutrients such as zinc, selenium, iron, copper, folic acid, and vitamins A, C, and E have been shown to modulate innate immunity, and their benefits have been explored in animal production. The inclusion of micronutrients in the diet of fish is among one of the most common strategies reported in the literature, highlighting the dietary inclusion of vitamins C and E. At appropriate levels, these vitamins may improve innate and adaptive immune responses, reduce mortality, and improve the animal's performance (Montero et al., 1999; Sahoo and Mukherjee, 2003; Wahli et al., 1998). However, in general, studies on neotropical freshwater fish have shown the use of different strategies and a broad range of experimental protocols make the comparison troublesome and the results controversial, as will be discussed below.

Garcia et al. (2007) fed pacu with diets supplemented with vitamin C (250 and 500 mg kg^{-1}) in combination with vitamin E (250 vitamin C + 500 vitamin E, 500 vitamin C + 500 vitamin E mg kg^{-1}) for 60 days, and then infected the fish with *A. hydrophila*. The vitamins did not influence survival, but vitamin C increased the number of thrombocytes in a dose-dependent manner. On the other hand, the postlarvae of pacu fed 750 mg of vitamin C kg^{-1} had a higher survival rate compared to those fed 125 mg kg^{-1} (Miranda et al., 2003). Abreu and Urbinati (2006) observed no effects of vitamin C on blood levels of cortisol, total protein, chloride, hemoglobin, leukocyte, and hepatic glycogen in matrinxã (*Brycon amazonicus*) fed for 60 days with diets supplemented with 100, 200, 400, and 800 mg kg^{-1}. However, pirarucu (*Arapaima gigas*) fed for 45 days with 800 mg vitamin C kg^{-1} had an increase in hematocrit, hemoglobin, the number of red and white cells, and the plasma total protein (Menezes et al., 2006).

Belo et al. (2005b) tested the effects of vitamin E (100 and 450 mg kg^{-1}) on the kinetics of macrophage recruitment and the formation of multinucleated giant cells (induced by a foreign body–glass coverslip) on pacu kept under different stocking densities (5 and 20 kg m^{-3}). Fish fed vitamin E (450 mg kg^{-1}) under a higher stocking density had lower cortisol levels and a higher number of macrophages, mast cells, and Langhans-like cells on the site of inflammation. In another report, Belo et al. (2014) fed pacu with vitamin E (12, 58, and 310 mg kg^{-1}) during 18 days and evaluated the inflammatory responses to a foreign body (glass coverslip) on connective tissue over 15 days. Fish fed with the lower vitamin E dose had a lower number of lymphocytes and LG-PAS $cells^{+}$ and an elevated plasma cortisol level, which was correlated to a lower number of monocytes on the site of inflammation. Using the same protocol but with a diet supplemented with 500 mg kg^{-1} of vitamin C, Belo et al. (2012) showed in pacu a higher number of macrophages and multinucleated giant cells on the site of inflammation and concluded that vitamin C enhances the macrophage activity on foreign-body inflammation. Petric et al. (2003) studied the inflammatory response in pacu fed for 40 days with 100, 200, and 500 mg kg^{-1} of vitamin C and showed that the vitamin increased the number of multinucleated giant cells in the site of inflammation. Further, Belo et al. (2005a) fed pacu with 100 or 400 mg kg^{-1} of vitamin E for 18 weeks and infected fish with the parasite *Anacanthorus penilabiatus* (Monogenea: Dactylogyridae). Fish fed vitamin E had a lower number of parasites on their gills.

Selenium (Se) is an essential micronutrient for antioxidant defenses, and one of its roles is to prevent the immunosuppression caused by oxidative stress. Takahashi et al. (2017) supplemented the diet of pacu with 0.3, 0.6, 0.9, and 1.8 mg Se-yeast kg^{-1} for 65 days and found that at 1.15 mg kg^{-1} (recovered concentration), the immune response and antioxidant

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defenses of fish were improved. The authors suggest that oxidative stress impairs the immune response and indicate the occurrence of a physiological trade-off between immune and antioxidant systems in this species. Castro et al. (2014) investigated the effect of the inclusion of chromium carbochelate in the diet of pacu (12, 18, and 36 mg kg^{-1}) on acute inflammation challenged by injection of inactivated *A. hydrophila* in the swim bladder. Trivalent chromium is known to reduce plasma cortisol levels in stress situations, and in this study, fish fed diets containing 18 and 36 mg of Cr kg^{-1} had plasma cortisol and glucose reduced and, indirectly, an enhancement of the immune response. In addition, Bortoluzzi et al. (2017) fed pacu for 105 days with chromium carbochelate and/or in combination with *Saccharomyces cerevisiae*. They observed that fish supplemented with chromium and *S. cerevisiae* had faster reepithelialization, a greater degree of organization of collagen fibers, and higher neovascularization when subjected to skin injury.

Probiotics and prebiotics

Probiotics are live microbial feed additives that modulate gastrointestinal microbiota, whereas prebiotics are nondigestible feed (carbohydrates or water-soluble fiber) that can stimulate the proliferation of beneficial gastrointestinal bacteria (Akhter et al., 2015). Both of these, at appropriate levels, improve production, health, and disease resistance in teleost fish (Bidhan et al., 2014; Lazado and Caipang, 2014; Nayak, 2010; Ringo et al., 2010a; Ringo et al., 2010b; Wang et al., 2008). Although their use is quite promising for aquaculture, few reports have investigated the effects of probiotics and prebiotics in neotropical fish.

Dias et al. (2012) fed matrinxã broodstock with *Bacillus subtilis* (10 g kg^{-1}) for nine months and observed that females had a higher number of oocytes as well as a higher fertility rate and larval survival during hatching. The same probiotic improved growth performance and survival while reducing cannibalism in matrinxã during the larval stage (Dias et al., 2011). The probiotic Efinol®L added in the water during transport of cardinal tetra (*Paracheirodon axelrodi*) reduced the cortisol level, the excretion of ammonia, and fish mortality after transport (Gomes et al., 2009). Mourino et al. (2012) fed a hybrid Brazilian catfish (*Pseudoplatystoma corruscans* x *P. fasciatum*) with a diet supplemented with 0.5% of inulin (prebiotic) or supplemented with the probiotic *Weissella cibaria* or a combination of these components. The combination stimulated beneficial gastrointestinal bacteria (lactic bacteria) and reduced pathogenic bacteria (*Vibrio spp* and *Pseudomonas spp*). In addition, this diet also increased the number of red blood cells and decreased circulating neutrophils. The probiotics *B. subtilis* and *S. cerevisiae* were tested in tambaqui (*Colossoma macropomum*), and both probiotics improved the feed conversion ratio and hematological parameters (da Paixao et al. (2017).

In a recent innovative study, Gallani et al. (2017) used *Rubrivivax gelatinosus*, a bacterium present in the environment successfully used for the treatment of fish industry effluent, to create a biomass rich in proteins and carotenoids. The authors fed pacu with diets supplemented with 0.5 or 1.5 g kg^{-1} of *R. gelatinosus* biomass during 60 days and showed that the *R. gelatinosus* biomass improved the immune response and growth parameters. This study reveals a promising use of a byproduct resulting from wastewater treating in aquaculture.

In conclusion, the use of probiotics and prebiotics in aquaculture is a fast-growing area and the increasing number of reviews published recently indicates their promising use (Akhter et al., 2015; Carbone and Faggio, 2016; Carnevali et al., 2017; Dawood and Koshio, 2016; Hai, 2015a, c; Hoseinifar et al., 2015; Huynh et al., 2017; Nawaz et al., 2018; Song et al., 2014; Wang et al., 2017). However, additional studies are necessary to understand their mechanism. Initially, it was hypothesized that probiotics are mainly active in the small intestine while prebiotics influence the microbiota of the large intestine in humans (Egerton et al., 2018). However, their effects converge to a central point that is the role of gut microbiota in the host immune system (Kelly and Salinas, 2017). Thus, understanding the effect of the gut microbiota on the overall animal health (i.e., cross-talking between microbiota and the immune system) seems to be the key to clarifying their exact mechanism. In addition, the prebiotics may also directly stimulate the innate immune system (Song et al., 2014). Hence, additional work is needed to investigate the ligand-receptor interactions, signal transduction pathways, and cytokines involved during direct immune stimulation by prebiotics (Hoseinifar et al., 2015; Song et al., 2014).

The immunostimulants

Immunostimulants can be defined as natural or chemical components that promote the activation of specific and/or nonspecific defense mechanisms (Anderson and Jeney, 1992; Ganguly et al., 2010). These components have been shown to be suitable for use in aquaculture (Sakai, 1999) to improve weight gain, feed efficiency, and/or disease resistance (Bricknell and Dalmo, 2005). Examples of immunostimulants are β-glucans (Chettri et al., 2013; Rodriguez et al., 2009), lactoferrin (Kumari et al., 2003), and levamisole (Hang et al., 2014; Maqsood et al., 2009). More attention in this section will be given to β-glucan because it is probably the most used and well-known immunostimulant for fish as well as to the growing use of

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medicinal plants in aquaculture. Although β-glucan may also be classified as a prebiotic, here, we decided to treat β-glucan as an immunostimulant because it has been shown to act in two different manners to improve animal health: first, as a typical prebiotic, stimulating the beneficial gastrointestinal bacteria, and second, directly stimulating the immune system through absorption in the small intestine. Consequently, the recognition of β-glucans as a fungus triggers a cascade of events resulting in immunostimulation, as recently discussed by Oliveira et al. (2019).

In one of the first studies with tropical fish, Sahoo and Mukherjee (2001) fed healthy and immunocompromised *Labeo rohita* for seven days with 0.1% of β-glucan and showed that β-glucan enhanced the innate and adaptive immunity and protection against bacterial infection in both fish groups compared with the control. The effects of β-glucan on innate immune responses and survival were also studied in pacu experimentally infected with *A. hydrophila* (Biller-Takahashi et al., 2014). The authors showed a higher survival rate in fish fed with β-glucan. Montoya et al. (2017) exposed matrinxã to a stressor and bacterial challenge after feeding with β-glucan and observed that β-glucan modulated the cortisol response prior to and after the stressor, increasing the number and activity of leukocytes. Moreover, cortisol showed to be an efficient modulator of both the humoral and cellular innate immune system by increasing lysozyme and complement activity as well as neutrophil and monocyte populations. In addition, Montoya et al. (2018) investigated the effects of two β-glucan molecules on the immune response of matrinxã prior and after the challenge with *A. hydrophila* and showed that both β-glucans affected the responses of fish by modulating the cortisol profile prior to and after the acute infection with *A. hydrophila*, and increased the mobilization and activity of leukocytes after infection. Diets supplemented with β-glucan were also tested in stress indicators in pencilfish (*Nannostomus trifasciatus*) during transport. β-glucan improved the ion balance during the transport (Abreu et al., 2014). The ion balance is considered an important stress indicator during transport in fish. Chagas et al. (2013) fed tambaqui, an important species in the northern region of Brazil, with diets supplemented with β-glucan (0.1%, 0.2%, 0.4%, and 0.8%) for 60 days and then experimentally infected the fish with *A. hydrophila*. The results demonstrated a higher survival rate of fish fed 0.1% of β-glucan.

Hisano et al. (2018) fed pacu with β-glucan (0.0%, 0.1%, 0.2%, 0.4%, and 0.8%) in combination with mannanoligosaccharides (MOS) for 30 days and found that the inclusion of 0.2% of β-glucans and MOS promoted the best growth response, feed efficiency, and intestinal morphology. Additionally, pacu fed a diet containing 0.2% and 0.4% of β-glucans and MOS exhibited significantly higher values of red blood cells than the control. The same product and concentrations (Glucan-MOS; 0.1%, 0.2%, 0.4%, and 0.8%) were tested in pacu by Soares et al. (2018), who showed that 0.2% and 0.4% diets were sufficient to increase the respiratory burst of leukocytes, lysozyme activity, and number of thrombocytes, neutrophils, and monocytes in the blood after a stressful handling and bacterial challenge. In addition, a reduced stress response was shown by decreased cortisol and glucose levels when compared to the control. Cerozi et al. (2017) tested the synbiotic combination of β-glucan and *B. subtilis* on the growth and immune response of pacu. Although the symbiotic treatment stimulated slight improvements in the intestinal microvilli, there was no effect on growth or immune responses in pacu.

Another immunostimulant that has been successfully used is levamisole, a synthetic anthelmintic compound widely used in mammals that has a potent stimulatory action on the innate immune system of fish (Kiron, 2012). Pahor et al. (2017) fed pacu diets containing levamisole (100, 150, 300, and 500 $mg\,kg^{-1}$) for 15 days and then submitted fish to air exposure and to an inoculation with *A. hydrophila*. Levamisole at 100 $mg\,kg^{-1}$ increased the leukocyte respiratory burst and activity of the complement system after *A. hydrophila* injection. Biller-Takahashi et al. (2016) also showed the positive effects of dietary levamisole (125 and 250 $mg\,kg^{-1}$) in the immune system of pacu fed for seven days and additionally demonstrated that levamisole may be used to promote adjuvant effects during immunization with *A. hydrophila* administrated intraperitoneally. In addition, Zanon et al. (2014) evaluated the effects of dietary levamisole for 60 days on the growth and immunological parameters of striped surubim (*P. reticulatum*), and although levamisole positively affected lysozyme concentration, no differences were observed in growth parameters.

In view of the large numbers of recent reviews, increasing attention has also been given to the use of medicinal plants (seeds, roots, flowers, and leaves) for disease control in aquaculture (Awad and Awaad, 2017; Bulfon et al., 2013; Carbone and Faggio, 2016; Citarasu, 2010; Dugenci et al., 2003; Galina et al., 2009; Hai, 2015b; Harikrishnan et al., 2011; Maqsood et al., 2011; Newaj-Fyzul and Austin, 2014; Reverter et al., 2014; Ringo and Song, 2016; Vallejos-Vidal et al., 2016; Vaseeharan and Thaya, 2014; Wang et al., 2017). Herbal biomedicines may present a new opportunity for the aquaculture industry (Citarasu, 2010) because they have environmental value due to their biodegradability (Yin et al., 2009). In addition, they can be easily obtained, are not expensive, and act against a broad spectrum of pathogens (Galina et al., 2009). In addition, they may also act as a growth promoter, are antistress, are appetite stimulators, and have been used to replace the animal protein in fishmeal (Awad and Awaad, 2017).

Several studies using the medicinal plant *Aloe vera* have been conducted in neotropical freshwater species such as matrinxã and pacu. *Aloe vera* is well known around the world due to its cosmetic and medicinal properties (Javed and Attaur, 2014). It contains more than 75 biologically active compounds with multiple biological activities such as wound

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healing as well as antibacterial, antiviral, antifungal, antidiabetic, and immune-modulatory properties (Akhtar et al., 2012; Choi and Chung, 2003; Christaki and Florou-Paneri, 2010; Hamman, 2008; Reynolds and Dweck, 1999). *Aloe vera* added to water (0.02, 0.2, and 2 mg of the powder L^{-1}) during the transport of matrinxã increased the leukocyte respiratory activity in a concentration-dependent manner (Zanuzzo et al., 2012). In addition, the authors observed *in vitro* higher respiratory activity in leukocytes of pacu incubated with the plant extract. *Aloe vera* bathing also improved physical and humoral protection in breeding stock after induced spawning in matrinxã. In general, fish bathed with *A. vera* had a higher number of epidermal goblet cells and an improved wound healing rate compared with the control after induced spawning (Zanuzzo et al., 2015). Recently, Zanuzzo et al. (2017) demonstrated that dietary *A. vera* for 10 days prior to transport and infection with heat-killed *A. hydrophila* improved the immune response in pacu. Specifically, *A. vera* prevented reductions in both leukocyte respiratory burst and the hemolytic activity of the complement system caused by transport. Further, fish fed *A. vera* also showed significantly higher leukocyte respiratory burst, serum lysozyme concentrations, and activity of the complement system in a dose-dependent manner 24 h after bacterial inoculation. Another well-known medicinal plant with immunomodulatory benefits is garlic, as mentioned before. The dietary inclusion of garlic (0, 1, 1.5, and 2 g kg^{-1}) for 15 days reduced the number of parasites in the gills of pacu infected with *A. penilabiatus* (Monogenea: Dactylogyridae), and after 45 days it increased the number of erythrocytes, leukocytes and thrombocytes while raising the hematocrit and hemoglobin concentration (Martins et al., 2002).

Abdel-Tawwab et al. (2018) fed African catfish (*Clarias gariepinus)* for 12 weeks with clove basil, *Ocimum gratissimum*, a plant native to Africa that grows in tropical and subtropical regions. The extract of clove basil significantly increased the intestinal villi length, villi width, and absorption area in a dose-dependent manner, and fish weight was highly correlated with these parameters. In addition, blood glucose and cholesterol levels decreased significantly while total protein, albumin, and globulin increased significantly in fish fed with the clove basil extract. Furthermore, antioxidants and immunity variables were significantly enhanced by clove basil extract supplementation, and fish mortality was significantly lower in fish fed with the extract in a dose-dependent manner after bacterial challenge.

In conclusion, the benefits of the immunostimulants are important in order to develop sustainable and environmentally friendly aquaculture. However, the efficiency of these components relies upon their dose/concentration, duration time, and route of administration, and studies to define the best protocol are required to optimize the benefits and avoid immunosuppression. Although some studies have shown a clear and direct concentration/dose-dependent stimulatory effect by medicinal plants, the mechanism of ligand recognition, extract composition, and activation of fish immune responses remains fragmented.

Future perspectives and concluding remarks

The knowledge surrounding stress and immune regulation in neotropical freshwater fish is still scarce and limited compared to cold-acclimated fish species such as salmon, trout, cod, and other well-known species. This is due in part to two main reasons: (1) the difference between the economies of tropical and subtropical countries, which impacts the knowledge of their respective native species, and (2) the fact that neotropical freshwater fish fauna is very rich compared to subtropical regions, comprising. 69 families and 5160 species (Reis et al., 2016) exhibiting an astonishing level of morphological, physiological, and behavioral diversity. Thus, the majority of the mechanisms discussed here regarding the neuroendocrine regulation of the stress and immune response were described in cold-acclimated species. Along these lines, and although some aspects of the stress and immune response are well conserved, a better understanding of the mechanisms of the neuroendocrine regulation of the stress and immune response in neotropical species is necessary. This advance will allow the development of specific and new strategies for the prevention and treatment of diseases triggered by stress at all stages of fish cultures for commercial production (Nardocci et al., 2014) as well for the conservation of the native stock population. Hence, the development of methods to assess the stress and immune responses of neotropical freshwater species is extremely important and highly welcomed at this point. For example, Abreu et al. (2009) and Biller-Takahashi et al. (2013b) adapted a method to evaluate the leukocyte respiratory burst activity in pacu, Biller-Takahashi et al. (2013a) developed a method to measure the serum bactericidal activity as an indicator of innate immunity in pacu, and Biller-Takahashi et al. (2013c) also developed a methodology to investigate the hemagglutination antibody titers in the same fish. In another study, Biller-Takahashi et al. (2012) standardized a technique to measure the hemolytic activity of an alternative complement pathway, which later was optimized by Zanuzzo et al. (2017). In addition, Zanuzzo et al. (2017) also optimized the method to measure the serum lysozyme concentration. These techniques have been successfully used and have significantly improved the knowledge about the modulation of immune response in neotropical freshwater species.

However, one of the current gaps faced by scientists in better understanding the biology of neotropical freshwater fish is the lack of whole-genome sequencing. Up to now, only one species considered as neotropical freshwater fish has had its

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genome sequenced: the blind cave fish (*Astyanax mexicanus*). Although the extensive use of zebrafish as an animal model has brought incremental knowledge about neotropical freshwater fish, the genome of other characidae species as well as specimens from the families Pimelodidae, Arapaimidae, and Cichlidae, among others would result in an exponential understanding of the biology of neotropical fish. In addition, the development of valuable screening tools such as transcriptome and proteome, as recently approached by Pimentel et al. (2016) and Mareco et al. (2015) for pacu and by Prado-Lima and Val (2016) for tambaqui, could bring a significant advance in the field of the stress and immune response of neotropical freshwater fish.

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Chapter 6

Evolution and physiology of electroreceptors and electric organs in Neotropical fish

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Introduction

Electrical gradients and the biological world are intimately related. There is no life without the selective separation of molecules and ions, and every living being, to a greater or lesser extent, generates electrical potentials as a consequence of its metabolic, biochemical, or biophysical processes. Electrical potentials of biological and nonbiological origin permeate essentially any environment on the planet. Therefore, it is not surprising that several groups of vertebrates, often independently, throughout their respective evolutionary processes, have developed the ability to detect fields by specialized cells and organs. In some fish, besides the organs to detect electrical gradients, there has also been the evolution of unique organs specialized in generating electricity. Among those, there are organs capable of generating enough power to serve as an attack and defense weapon, but in the vast majority of species, the electricity generated is weak and much more relevant for communication and for detection of other organisms and objects.

Here, the term electroreception is being used to define an organism's ability to detect electrical gradients in the environment by means of specialized sensory organs, called electroreceptors. Likewise, (bio)electrogenesis indicates the ability to generate electric discharges associated with the presence of a specialized tissue, the electric organ (EO), whose cellular unit is the electroplate or, in more recent literature, the electrocyte (Chagas and Paes de Carvalho, 1961; Bullock and Heiligenberg, 1986).

If we consider that (nonpure) water is a conductive medium, it is not difficult to accept that electroreceptors may have become an adaptive advantage for aquatic organisms because this type of biosensor would allow the organisms to detect an entirely alternative universe of stimuli and, consequently, explore the habitat where they live in a differentiated and probably more efficient way. Obviously, however, this advantage can only be attained if the electroreceptors are associated

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with a nervous system capable of processing such information. With the emergence of vertebrates and craniates in the aquatic environment more than 600 million years ago, sensorial structures physiologically specialized in detecting electric gradients in the environment also came into existence. These structures (electroreceptors) were capable of detecting electrical potentials of both biological and nonbiological origin. Consequently, electrosensitive animals could better distinguish the properties of the habitat where they lived, including eventual fields generated by other organisms such as conspecifics, prey, and predators.

In this chapter, we will discuss evolutionary, anatomical, and physiological aspects related to electroreception and electrical organs in Neotropical fish. In order to gain a better perspective and understanding about the presence of such specialized structures in these fish, it is worthwhile to address their occurrence under a broader phylogenetic perspective.

Phylogenetic occurrence of electroreceptors and electrical organs in vertebrates

As the knowledge about the phylogenetic relationships between vertebrates and especially among fish is improving fast, it is possible to understand more precisely the various evolutionary events related to the emergence (and disappearance) of electroreceptors and EOs. There is a substantial amount of evidence that electroreceptors and EOs have been "invented" multiple times in the evolutionary history of vertebrates in general, and within fish in particular (Alves-Gomes, 2001; Bullock et al., 1983). The understanding of these processes is a topic that has attracted considerable interest within evolutionary and comparative biologists, especially because the morphological and physiological specializations associated with these specialized cell types and organs reflect diverse nonhomologous evolutionary processes. In other words, the process of natural selection, in the case of electroreceptors and EOs, has produced final results with an appearance and function that are extremely similar, through completely different pathways.

In order to better contextualize the emergence of these organs in vertebrates, it seems instructive to initially address the basal phylogeny of the chordates, especially because electroreceptors can be found very early in vertebrate evolution. The Phylum Chordata (animals with a notochord) consists of three main clades: Cephalochordata (amphioxus), Urochordata (tunicates), and Craniata (animals with a cranial box). The latter, in turn, can be divided into three main groups: the hagfish (Mixini), the lampreys (Cephalaspidomorphi), and the Gnathostomata (all other craniates with jaws, from cartilaginous fish to humans). Electroreceptors appear, as an evolutionary novelty, among the early craniates. In fact, except for the hagfish, electroreceptors are found in almost all the major lineages within the craniates, including the lampreys, all the cartilaginous fish (Chondrichthyes), the coelacanth (Coelacanthimorpha), the lungfish (Dipnomorpha), some amphibians, and the basal actinopterygians (Cladistia and Chondrostei) (Fig. 6.1 and see Chapter 1).

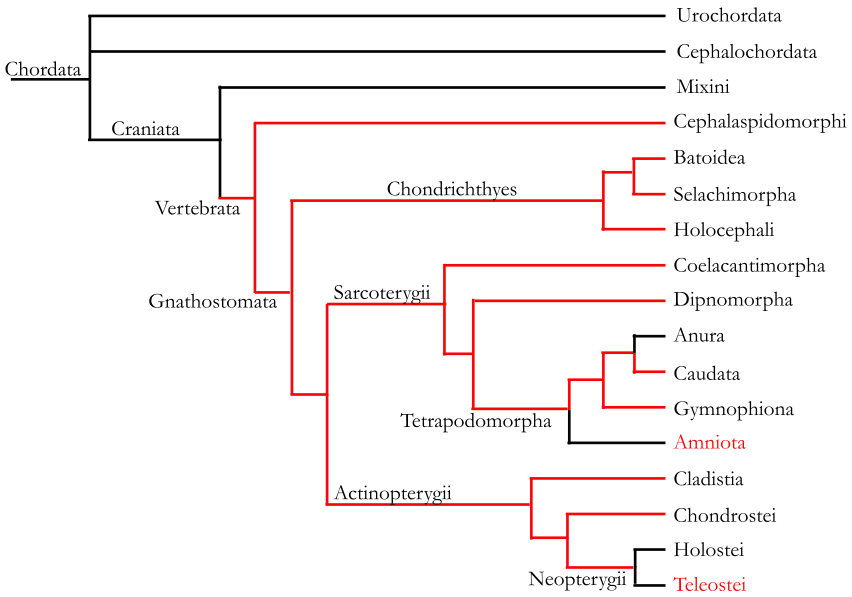

FIG. 6.1 Occurrence of electroreceptors in vertebrates. The phylogenetic relationships and taxonomic hierarchy of this chapter follows Chapter 1. Electroreceptor organs, coded by the *red* branches and names in the figure, are found in basically all lineages of aquatic vertebrates, but disappeared in the two lineages of Tetrapodomorpha that fully invaded the terrestrial environment (Anura and Amniota) very likely due to the fact that atmospheric air is electrically insulating. Electroreceptors also disappeared in the basal clades of the Neopterygian fishes, including the basal teleosts (Holostei and Teleostei). The reason for this second disappearance is not well understood, but perhaps not coincidentally, it conforms with the transition of fishes from marine (highly conductive) to fresh (less conductive) waters. These specialized sensorial organs were "reinvented" in some isolated groups of teleosts, as well as in few mammals that have their life cycle associated to water or humid places (see text and Fig. 6.2). These punctual appearances are represented in the figure by the names in *red* (*gray* in the print version).

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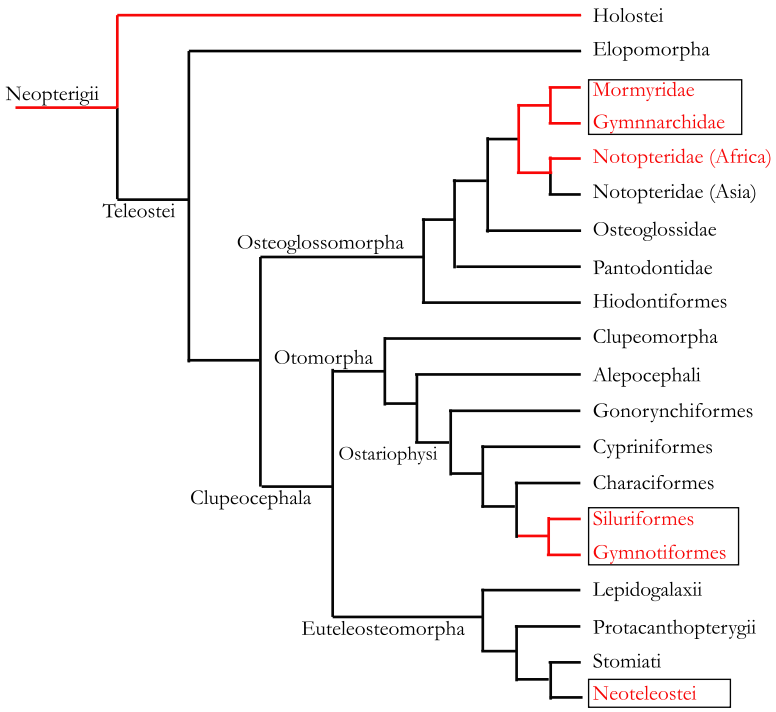

FIG. 6.2 Electroreceptors and electric organs in teleosts. The large majority of teleosts do not possess either electroreceptors or electric organs (*black* branches in the figure), but electroreceptor organs appeared as an evolutionary novelty at least twice within the group: in the common ancestor of the families Mormyridae and Gymnarchidae (African electric fish) and in the African (but not Asian) notopterids. Then, again, in the distantly related common ancestor of the catfishes (order Siluriformes) and the South American electric fishes (order Gymnotiformes). The presences of electroreceptors are indicated by the *red* branches in the figure (*gray* in the print version). It is not clear if electroreceptors were lost in the Asian notopterids or if they appeared independently only in the African notopterids. The lack of electroreceptors in the majority of teleosts reinforces the idea of nonhomology between teleost x nonteleost receptors, but also indicates the lack of homology between the electroreceptors present in the African and in the South American electric fishes. Electric organs are found in all the Mormyridae and Gymnarchidae, in few catfish families (Siluriformes), in all the Gymnotiformes and in two perciform genera (*Astroscopus* and *Unanoscopus*) within the Neoteleostei.

For some reason not yet well understood, electroreceptors seem to have "disappeared" in the basal neopterygeans, that is, in the Holostei (Lepisosteiformes and Amiiformes) and in the basal lineages of modern teleosts (Fig. 6.2). Later in time within the teleosts, these specialized organs appeared again, several times in the catfish (Order Siluriformes), in the South American electric fish (Order Gymnotiformes), the African electric fish (Order Mormyriformes), and in the African but not the Asian notopteriforms (Alves-Gomes, 2001; Braford, 1982; Bullock et al., 1982; Fields et al., 1993; Iggo et al., 1998).

For the sake of clarification and accuracy, it is worthwhile to explain in more detail the presence of electroreceptors within the Sarcopterygii, the lineage grouping the Coelacanthiformes (coelachant), the lungfish (Ceratodontiformes), and the Tetrapodomorpha (the lineage leading to terrestrial vertebrates, including man). Within this group, electroreceptors are found in all representatives of the former two clades, which are aquatic, but also in a few representatives of terrestrial vertebrates. Electroreceptor organs disappeared in frogs and toads from the Order Anura and in the Amniota, that is, the lineage of vertebrates that invaded the terrestrial environment (Fritzsch, 1981; Fritzsch and Wahnschaffe, 1983; Fritzsch and Wake, 1984; Himstedt and Fritzsch, 1990; Munz et al., 1982; Northcutt et al., 1995; Roth and Schlegel, 1988), see Figs. 6.1 and 6.2. It is believed that the main reason for the absence of electroreceptors in land vertebrates is the fact that the atmospheric air is electrically insulating, so that electrical signals do not propagate through terrestrial environments.

Within the Amniota, it is currently known that electroreceptor organs are present in the two mammalian groups of the order Monotremata: the platypus (*Ornithorhynchus anatinus*) and the two genera of echidnas (*Tachyglossus* and *Zaglossus*), all inhabitants of Australia and New Guinea (Andres et al., 1991; Gregory et al., 1987, 1989; Manger et al., 1997; Manger and Pettigrew, 1996; Pettigrew, 1999; Scheich et al., 1986). These findings are not completely surprising because these animals have their respective life cycles associated with wet or aquatic environments where they live and forage. Another mammal living in humid and semiflooded areas that responded positively to electrical stimuli simulating the electric field of its prey (earthworms) was the star-nosed marmot, *Condylura cristata* (Gould et al., 1993), which inhabits the East of North America. The electroreceptors of these animals are primarily associated with food detection in electrically conductive environments. To complete the list of mammals, the presence of electroreceptors was also reported in dolphins of the species *Sotalia guianensis* (Czech-Damal et al., 2012; Liebschner et al., 2007). This species occurs in the coastal waters of Northeastern South America, and is considered the sister species of *Sotalia fluviatilis*, which inhabits the Amazon Basin. If the physiological functionality of these electroreceptors is confirmed in *S. guianensis*, it will be

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interesting to verify not only whether the freshwater species also has electroreceptors, but also to compare their anatomical, physiological, and behavioral properties.

As we start to inventory organs specialized in generating electricity (EOs), it is possible to notice that first, the occurrence of EOs is far more restricted than that of electroreceptors and they only occur in a few fish groups. In fact, to date EOs have been described only for a few marine rays of the orders Torpediniformes and Rajiformes, and for representatives of four groups of teleosts: all the South American electric fish (Gymnotiformes), all the African electric fish (Mormyriformes), some genera of catfish (order Siluriformes) from Africa and Asia, and two genera of the order Perciformes (*Uranoscopus* and *Atroscopus*) (Fig. 6.2).

As in this chapter we will focus on the physiology and functioning of electroreceptors and electrical organs in groups of fish restricted to the Neotropical region, the list of taxa examined will include the cartilaginous fish (rays, sharks, and chimeras of the Class Chondrichthyes); the South American lungfish (order Lepidosireniformes); the lampreys (order Petromyzontiformes) because the genera *Geotria* and *Mordacia* complete their respective life cycles in the Southern portion of South America; the catfish (order Siluriformes); and the South American electric fish (order Gymnotiformes). In addition to these groups, we will also consider the only two genera of marine perciforms of the family Uranoscopidae that inhabit the coast of South America and also have electrical organs.

Passive and active electroreception

When the term electroreception is employed, normally a complex network of sensorial structures and brain nuclei are being referenced, which interconnects peripheral sensory cells highly specialized and sensitive to voltage gradients, to nuclei and nerve centers at different levels in the cerebral hierarchy. These neuronal populations in the central nervous system (CNS) process the information originated in the periphery (electroreceptors), as the sensorial cells of the electroreceptor organs modify their respective discharge patterns as a function of the modulations in the electric fields present in the water, allowing the fish to extract information about the electrical landscape of the surrounding environment. Fields of biological as well as nonbiological origin compose the aquatic electrical landscape, and different types of electroreceptors (see below) are tuned to encode different aspects of this landscape. The integration in the CNS of the physiological activity from different groups and types of electroreceptors gives the fish the possibility of perceiving details of what exists and what is happening in the surrounding world. The network of electroreceptors, in fish, is generally scattered throughout the body surface, but it is concentrated in the head region.

When fish only detect electric gradients generated by external sources, that is, without the presence of an EO, the sensory modality is called *passive electrolocation*. This type of electroreception is used for orientation, detection of prey and predators, and navigation; it has also been demonstrated for sea rays in the detection of reproductive partners (Brown, 2002; Kalmijn, 1974, 1982; Sisneros et al., 1998; Tricas et al., 1995).

A different situation is found when, in addition to electroreceptors, fish also possess an EO. By means of their electrogenic tissue, fish more or less constantly generate an electric field around themselves, and simultaneously monitor the properties of the field through the network of electroreceptors in the skin. In this case, instead of detecting electrical signals originated externally, individual fish detect the interference caused by objects or organisms with electrical conductivity different from the water, in the lines of force of the self-generated field. This situation implies the joint action of two independent systems: an electrogenic motor system, the EO, and a second formed by the sensory organs, the electroreceptors. Because this sensory modality depends directly on the electric organ discharges (EODs), it is called *active electroreception* (Bastian, 1981, 1986; Caputi et al., 2008; Carr and Maler, 1986; Heiligenberg and Bastian, 1984; Von der Emde, 1999).

The combined action of the electrogenic and the electrosensory systems (EES) defines what in this chapter will be called the electrogenic-electrosensory system, or EES, as substantiated by Alves-Gomes (2001). The EES is intimately associated with active electrolocation and fish use it not only for the same tasks listed for passive electroreception, but also for a fundamental additional function: intra- and interspecific communication (Collin and Whitehead, 2004; Hopkins, 1999; Perrone et al., 2009; Stoddard, 1999, 2006).

Evolutionary aspects of the EES

As we examine in detail the occurrence of electroreceptors and electrical organs in fish, it is possible to notice that not all electroreceptive fish have electrical organs. This comes as no surprise because the electrosensory system provides the organism a complete sensory channel in itself, such as vision or smell, through which fish can extract crucial information about the environment. However, the inverse situation, that is, an electrogenic fish that does not have electroreceptors, is much more difficult to understand from the evolutionary point of view. This condition, i.e., a fish that is capable of generating an electric field but not able of detect it, although unusual, is found in the genera *Astroscopus* and *Uranoscopus*, two

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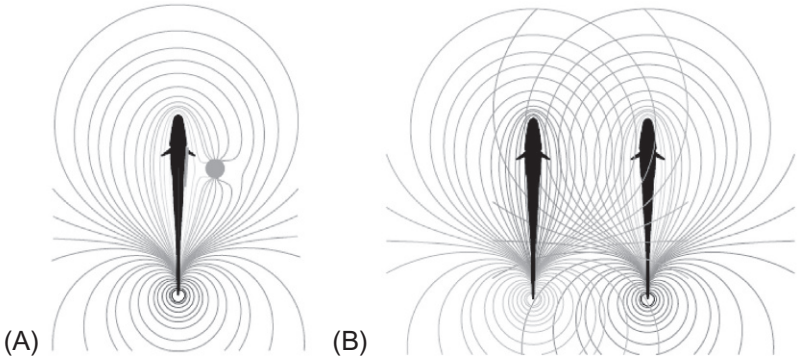

FIG. 6.3 Functioning principle of the electrogenic and electrosensory system (ESS). In A, during an EOD, an electric field is generated around the fish, with isopotential lines decreasing in force as a function of the distance from the fish. If an object with an electrical conductivity larger than the water (like a metal cylinder in A) is placed near the fish, the line forces will bend toward the less resistive pass, that is, through the metal cylinder. If the object is more resistive than the water, as the plastic cylinder, the line forces will diverge from the object. In both situations, the strength of the field in the fish's skin near the object will change, and the electroreceptors located in this area will detect these changes, delivering the information to higher centers in the nervous system. In B, the interference is caused by the EOD of another fish. Thus, fish with active electroreception can locate objects and other organisms as well as communicate, even in the absence of light. *Adapted from Krahe, R., Maler, L., 2014. Neural maps in the electrosensory system of weakly electric fish. Curr. Opin. Neurobiol. 24, 13–21.*

marine perciforms of the Family Uranoscopidae that have electrical organs but not electroreceptors neither nerve centers dedicated to electroreception (Bullock et al., 1982, 1983), It is thought that the EO in uranoscopids could serve to stun the prey or to avoid predation as their discharge may reach tens of volts (<50 V), but no specific physiological or behavioral studies are currently available on the subject. With the exception mentioned above, electrical organs have always evolved within groups of fish that were already electroreceptive.

Because electrical organs and electroreceptors are not always present concomitantly in fish, and because there is sufficient evidence pointing to a homoplasic appearance of both electroreceptors and EOs in the evolutionary history of fish (Alves-Gomes, 2001), in this chapter we will reinforce the view that these two systems are evolutionarily independent and, therefore, compose separate organismal and functional unities. When there is the simultaneous presence and synchronous functioning of electrical organs (motor side) and electroreceptors (sensory side), a novelty condition is achieved that is known to be present in a small fraction of fish groups. These two distinct systems and their respective brain structures merge to create a separate, third condition, the EES. As mentioned before, the EES is the fundamental anatomical and physiological basis for active electroreception. The basic operating principle of the EES can be seen in Fig. 6.3.

Electrical landscape: Current and voltage sources in aquatic environments

Living organisms are not the only sources of electrical potential in the environment. Although biological systems and electricity are intrinsically associated because electric potential will exist whenever there is a separation of electrical charges or ions, electrical gradients can also be generated by electrochemical differences between different types of substrates and the water, physicochemical discontinuities, electromagnetic phenomena, and geomagnetic anomalies, to name the main ones. Therefore, the resultant electrical landscape of the given ambient is a complex blend of electrical "signals" originated by all the possible sources mentioned above. Fish possessing only electroreceptor organs, that is, without the EO, can also extract important information about the physical characteristics of their habitat once their receptors have enough sensitivity to detect the weak fields around them.

Perhaps the most impactful sets of studies about the detection of electric fields/landscapes by fish are the classical papers published by Adrianus Kalmijn in the 1970s and 1980s, when the author demonstrated that several electroreceptive fish possess electroreceptors with functional thresholds below the strength of the biological and nonbiological fields present in their ambient. Further, Kalmijn demonstrated that marine cartilaginous fish have electroreceptors sensitive enough to orient themselves in relation to the Earths' magnetic field while either swimming or drifting in aquatic currents (Kalmijn, 1971, 1974, 1988a,b).

In organisms, the semipermeable cell membrane is the main element responsible for the separation of charges in different cell types, from plants to vertebrates (Ansari and Bowling, 1972; Kandel and Schwartz, 1985; Yan et al., 2009). These fields may vary by several orders of magnitude in their strength, ranging from 10^{-9} V/μm, such as those reposted for microorganisms (Gow, 1989), to discharges of more than 600 V as generated by the electric eel (*Electrophorus electricus*), the most powerful (and dangerous) known electric discharge of biological origin. In vertebrates, the activity of individual nerve and

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muscle cells generates electrical potential that may reach 120–150 mV (Kandel and Schwartz, 1985). Depending upon the number and timing of the involved cells, the voltage can be summed up, and groups of cells working in synchrony produce fields with values proportional to the number of cells firing. Further, organisms are not completely impermeable and, especially in water, ions may flow between the internal tissues and the environment through the semipermeable membranes as a result of electrochemical gradients. In fish, the semipermeable mucosa in the gills, mouth, pharynx, and perianal area are places where the flow of ions is relatively continuous. At these sites, careful measurements of electrical potential reveal fields of direct current that can reach 500 μV (Kalmijn, 1974, 1988a,b). Further, to cite other relevant values in fish, the voltage gradient measured from a 15 cm long marine sole *Pleuronectes* reaches 200 μV/cm at 0.5 cm from the subject, and 0.2 μV/cm at a distance of 10 cm (Kalmijn, 1974).

Krajew (1957) reports that fields generated by water filtration in river banks can generate fields between 10 and 20 mV/m and fields associated with water flux in the river can vary between 19 and 30 mV/m. Peters and Bretschneider (1972) cite similar numbers, up to 15 mV/m for hydroelectric fields, exhibiting a high relative constancy in both direction and force. Large-scale oceanic surface current measurements also have been made and the numbers found were of the order of 0.05–0.5 μV/cm (Kalmijn, 1974). Other measures mention values between 0.08 and 0.16 μV/cm in oceanic waters (von Arx, 1962) and 0.25 μV/cm in coastal channels (Kalmijn, 1974).

Tidal forces, tectonic processes, magmatic events, temporal variations in the strength of the Earth's magnetic field due to magnetic storms, and other related geomagnetic phenomena can also generate more or less stable electric fields on the planet, including the aquatic ambient. Although the effects of these types of fields are much more difficult to measure in fish, they are part of the universe that can be detected by eletroreceptors. As an interesting example, in 1933, the scientific journal Nature published a note revealing that two Japanese researchers, Drs. S. Hatai and N. Abe, observed that catfish changed their behavior in laboratory tanks about 6 h before a major seismic event (earthquake). Later, related works were published (Hatai and Abe, 1932; Hatai et al., 1932) and, as a result, specific studies were done to test catfish as potential seismic detectors (Ikeya et al., 1996). Two main possibilities could be associated with the observed sensitivity of the fish: because the catfish are electroreceptive, they would either be detecting disturbances in the electromagnetic field of the Earth or the fish could also detect small preearthquake seismic disturbances through the lateral line. It is probable that both mechanisms mentioned above are operating simultaneously, but the important aspect of it is that the physiological thresholds of catfish electroreceptors, and several other electroreceptive fish as well (Kalmijn, 1974), are low enough that they can detect such types of electromagnetic anomalies.

Another potentially relevant but less studied source of electromagnetic interference in the water is the fields generated by electricity transmission lines passing nearby aquatic environments. It has been shown (Basov, 2007) that high-voltage lines can generate electric potentials in the water on the order of 10–400 μV/cm, a value fully compatible with the sensitivity of teleost electroreceptors.

One last source of electromagnetic noise in the water is the atmospheric lightning during storms. Although the effects of this type of event have not been studied, they can be detected by electroreceptors of several fish species in both marine and freshwater environments (Hopkins, 1973).

In summary, most of the field values reported above are perfectly within the detection limits of the various fish electroreceptors (see below). Therefore, electroreceptive fish can detect the several components that make up the electrical landscape of the habitat where they live and, in this way, extract additional and crucial information about the changes in the space they occupy.

Electroreceptor types and their basic physiological properties

According to their respective anatomical and physiological characteristics, electroreceptors can be subdivided into several types and subtypes, but there are two basic functional categories: ampullary and tuberous electroreceptors. These two types of electroreceptors have a complex evolutionary history and both have evolved multiple times, independently, in the history of vertebrates, or even within the teleosts alone (Alves-Gomes, 2001; Zakon, 1986). Electroreceptors are sensorial *organs*, and each organ is formed by several types of cells that confer specific characteristics to the different types of receptors. Among the cells that make up the electroreceptors are the sensory electroreceptive cells, which are the real sensors of the organ from a physiological and functional point of view. Throughout the present text, the term electroreceptor is used as a synonym for electroreceptor organ.

Ampullary electroreceptors are characterized by having a pear-shaped structure (ampulla) connected with the external media by means of a tube that has a pore at its distal end, on the surface of the skin (Fig. 6.4). At the base of the organ, at the bottom of the chamber, there is a sensory epithelium where the receptive, ciliated (electro)receptor cells are supported by supporting cells (Heiligenberg, 1993; Szabo, 1974). The length of the tube varies greatly depending on the species. In rays, it has been noticed that the canal is longer in marine than in freshwater species due to the greater electrical conductivity of

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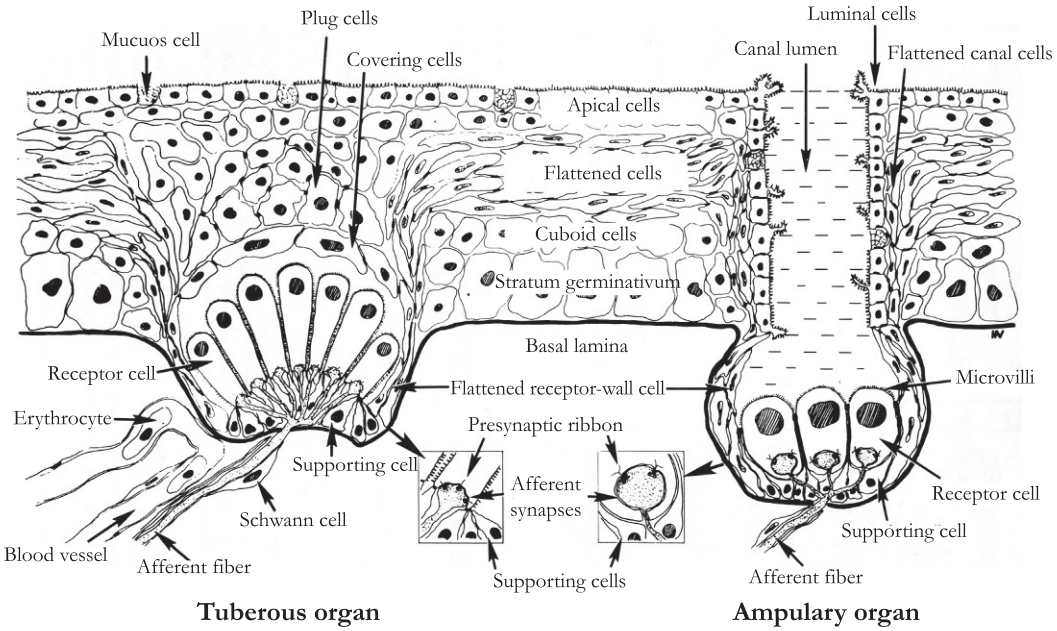

FIG. 6.4 Schematic showing the two basic types of electroreceptors found in fish, here exemplified in the gymnotiform genus *Eigenmannia*. On the left side is a tuberous electroreceptor, separated from the external medium by an epidermal plug of cells. These receptors are only found in fish that also possess an electric organ (with only one exception—see text) and have their higher sensitivity for frequencies around the dominant frequency of the fish's own EOD. On the right, an ampullary organ, which connects to the external medium by means of a canal. These receptors are most sensitive to direct current electric fields or low frequency (<15 Hz) alternated current fields. *Adapted from Heiligenberg, W., 1993. Eletrosensation. In: Evans, D.H., (Eds.), The Physiology of Fish. Marine Science Series. CRC Press, Boca Raton, pp. 137-160.*

the salt water in relation to the internal salt content and skin resistance of the fish (Kalmijn, 1974; Szabo et al., 1972); see a more detailed explanation below.

There is also strong evidence to postulate that ampullary receptors of marine species tend to be more sensitive than those of freshwater fish. Peters et al. (2007), after an extensive review of available data, established the detection limit of ampullary receptors of 1 μV/cm for freshwater and 20 nV/cm for marine species.

The tube of the ampullary receptors is filled with a mucous substance of very low electrical resistivity while the wall of the ampulla is highly resistive, being composed of flattened cells that interconnect by means of tight junctions and desmosomes (Jørgensen, 2005). Ampullary electroreceptors respond to stimuli of direct or near direct current (<0.1 Hz) or alternating current of low frequencies (usually <10 Hz), but can reach up to 50 Hz depending on the species (Bodznick and Montgomery, 2005; Zakon, 1986). From an embryological point of view, these low-frequency electroreceptors are believed to have derived from the neural plaques that will form the ciliated cells of the lateral line and other mechanoreceptors in vertebrates (Northcutt et al., 1995; Ronan and Bodznick, 1986). All known ampullary electroreceptors are enervated by nerve fibers from the lateral line system, which reinforces this hypothesis (Bodznick and Montgomery, 2005; Northcutt, 1997, 2005; Zakon, 1986).

Nevertheless, significant differences are found between the ampullary receptors of teleosts and nonteleosts. So far it has been assumed that the ampullary electroreceptors found in nonteleost fish (in this chapter: lampreys, cartilaginous fish, and lungfish) are homologous. Considering the phylogenetic hypotheses of vertebrates (Figs. 6.1 and 6.2), it is clear that electroreceptors appeared as a synapomorphy of Vertebrata. Further, nonteleost ampullary electroreceptors, despite having a diverse anatomy (Jørgensen, 1982, 2005), have very similar physiological and functional principles. For instance, all nonteleost ampullary receptors are excited by cathodic stimuli (when the lumen of the ampulla becomes negative relative to the base of the sensory cells) and inhibited by anodic stimuli (when the lumen of the ampoule becomes positive). The teleost electroreceptors (in this chapter represented by catfish and gymnotiforms), on the other hand, function inversely and are stimulated by anodic stimuli (Bodznick and Montgomery, 2005). This difference appears to reflect quite clearly the nonhomology between the ampullary receptors of teleosts and nonteleosts. Further, as previously mentioned, the ampullary receptors in fact disappeared in the basal lines of teleosts, to be reinvented again independently in a different, distantly related group of teleosts (Alves-Gomes, 2001; Fig. 6.2).

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The nerve fibers innervating the ampullary electroreceptors, even in the resting state, have an intrinsic discharge rate, generating action potentials in a constant manner. Depending on the type of stimulus, whether excitatory or inhibitory, these cells either accelerate or decrease their respective discharge rates. A detailed review of the organization and physiology of the electrosensory system in fish can be found in Bennett (1971b), Bodznick and Boord (1986), Bullock (1982), Carr and Maler (1986), Finger (1986), Northcutt (1986), and Szabo (1974).

Tuberous electroreceptors, on the other hand, have a rather distinct anatomy and physiology in relation to the ampullary ones. These receptors are physiologically adapted to detect electrical signals generated by electrical organs and, so far, have only been described in electrogenic teleosts, with one single exception (see below). Tuberous receptors are sensitive to alternating electric signals, having the highest sensitivity in the same range of the fundamental frequency of EODs of the respective species that possess them, which can range from less than 50 to almost 2000 Hz.

The view that tuberous electroreceptors would be found exclusively in teleost fish possessing electrical organs, that is, in the South American (order Gymnotiformes) and the African electric fish (order Mormyriformes), was undisputed until near the end of the 1980s. However, in 1988, Andres et al. (1988) described the anatomy of a tuberous electroreceptor for an unidentified species of the South American catfish of the genus *Pseudocetopsis*. No subsequent behavioral or physiological studies were carried out with these fish and the ecological and behavioral functions of these electroreceptors in *Pseudocetopsis* are still to be unraveled. The taxonomy and phylogeny of the family Cetopsidae is still not well resolved and, consequently, the systematic relationship of the fish examined by Andres et al. (1988) within the family is uncertain. It would be very interesting to know, first, which species was actually used by the authors and, second, whether tuberous electroreceptors are also present in other species within the genus or in other genera within this family (Andres et al., 1988; Sullivan et al., 2006).

The genus *Pseudocetopsis* contains examples of fish with reduced or absent vision. In theory, the presence of tuberous electroreceptors could benefit these fish with the ability to detect the discharges of Gymnotiformes, with whom *Pseudocetopsis* can live in sympatry, and perhaps other sources of electric fields of the environment where they live. Aside from this only exception, tuberous electroreceptors are always associated with active electroreception. These sensory organs constantly monitor the properties (amplitude and timing) of the electric field generated during an EOD and, thus, are fundamental for localization and communication.

Anatomically tuberous electroreceptors do not have a duct connecting the sensorial epithelium to the surface of the skin. Instead, these organs have a plug composed of epidermal cells that are not very firmly packed, and the sensory cell is exposed in an intraepidermal cavity (Jørgensen, 2005; Szabo, 1974). The sensory cell of the receptor organ makes synaptic connections with afferent nerve fibers whose cell bodies lie in the lateral line nerve ganglia (Fig. 6.4). The basal membrane of the sensory cell is excited when current flows from the outer environment into the fish body, that is, from the outside inward, and each nerve fiber can innervate between one and dozens of tuberous organs. As a result of such an innervation pattern, the spatial reception field of a single fiber can be large, which, in turn, gives the electrosensory system a spatial representation not as sharp as, for example, optical images in the retina (Kawasaki, 2005). Due to their respective physiologies, tuberous electroreceptors can be subdivided into at least two basic types: one class of electroreceptors produces a single action potential for each detected EOD and thereby encodes temporal aspects of EODs. This class is called time coders (or "T-units"). A second type of tuberous electroreceptor is sensitive to changes in the amplitude of the electrical signal ("amplitude coders" or "P-units"), and generates more action potentials with a greater signal amplitude.

The embryological origin of tuberous electroreceptors in teleosts is still not well established and two hypotheses predominate. In the first, under one interpretive line these electroreceptors are induced in the ectoderm by the nerves of the lateral line that innervates these cells (Vischer, 1989, 1995; Vischer and Heiligenberg, 1989). In another view (Northcutt, 2005; Northcutt et al., 1995), electroreceptors arise from the neuroplates that will generate the cells of the lateral line ("dorsolateral placodes"). Details about the physiological and anatomical aspects of tuberous electroreceptor organs will be addressed in more depth when addressing each fish group specifically.

EOs, EODs, and their physiology

For the large majority of cases studied, electric organs in fish are specialized tissue embryologically derived from muscles (Kirschbaum, 1977, 1983; Kirschbaum and Schwassmann, 2008). Different groups of fish have EOs derived from different muscle precursors, and the basic physiological principles driving muscle action are also found in EOs. In the EO, the cellular unit (the electrocyte) has lost its ability to contract as in a regular muscle cell and, when depolarized by a motor synapse, each electrocyte produces an action potential of about 110 mV. The synchronous depolarization of the electrocytes produces what is called the EOD. When a pair of metal probes are placed near an electric fish, the discharge of the fish's electric organs become strong enough to be captured by a relatively simple electronic apparatus, connecting the probes to an

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audio amplifier. This amplified signal can be sent to a speaker or recorded. Literally, one can listen to the fish's EODs when they are in their natural habitats. This is the principle of an "electric fish detector." EODs can also be digitized and used as powerful tools in behavioral and systematic studies of electric fish. Different species produce different EODs and, within the same species, the temporal and biophysical properties of the discharges vary between males, females, and subadults, especially during the reproductive season. Even in the same individual, EODs may change depending on the physiological state and hormone levels at time windows ranging from hours to months (Allee et al., 2009; Alves-Gomes, 2001; Crampton et al., 2008; Dulka and Maler, 1994; Lovejoy et al., 2010; Markham et al., 2009; Rodríguez-Cattaneo et al., 2008; Salazar and Stoddard, 2009; Silva et al., 2008; Stoddard, 2006; Stoddard et al., 2006, 2007; Zakon, 1993, 2005). The diversity and variability of the electrical signals among electrical fish allow the EODs to become a crucial tool also for several types of behavioral, populational, and evolutionary studies.

EOs derived from muscle tissue (myocytes) are said to be myogenic. Among all known electrogenic fish, there is only one exception to this condition. The fish of the family Apteronotidae (order Gymnotiformes) have a myogenic organ only during the first days of life, during the larval stage. However, early in the ontogenetic process, the myogenic organ is reabsorbed and the motor neurons themselves, those that initially innervate the primordial EO, become the cellular unit of the electrogenic tissue (Kirschbaum, 1977, 1983; Kirschbaum and Schwassmann, 2008). In this case, the EO of the apteronotids is said to be neurogenic.

EOs usually discharge under a neural command initiated in a motor nucleus in the CNS. The most well-known circuit among Neotropical fish is that of gymnotiform fish. In this group, each EOD is triggered by a command started in the pacemaker nucleus located in the fish's medulla. Until relatively recently, this nucleus was known to possess only two types of cells: pacemaker cells and relay cells. Pacemaker cells have a constant, intrinsic physiological activity and are interconnected by means of gap junctions, causing them to function synchronously. Even when isolated in a Petri dish with physiological solution, the pacemaker cells continue discharging at their normal rate for several hours (Moortgat et al., 2000a,b). By means of electrical and/or chemical synapses, the pacemaker cells connect with the relay cells (Dye and Meyer, 1986) and these, in turn, send their axons out of the nucleus; they then contact the spinal motor neurons that innervate the EO (Elekes and Szabo, 1981, 1982, 1985; Ellis and Szabo, 1980; Kawasaki and Heiligenberg, 1988; Kawasaki et al., 1988). Thus, each EOD is initiated in the pacemaker nucleus, and for each action potential of the pacemaker cells, there is an action potential of the relay cells and, at the end of the chain, a discharge of the electrical organ. Pacemaker cells fire about 0.5–0.6 milliseconds (ms) before relay cells and these, in turn, fire about 3–5 ms before the EO (Dye et al., 1989; Dye and Meyer, 1986; Fig. 6.5). Through electrical or chemical stimulation of the pacemaker, it is possible to induce modulations in the rhythm of EOD of the gymnotiforms. This circuit has been a very important experimental model for neurobiological studies, especially in neuroethology.

A third type of cell (parvocell) has been described in the pacemaker of the gymnotiform electric fish genus *Apteronotus* (Smith et al., 2000), but despite its morphology and differentiated histochemical patterns, the physiology and function of this new cell have not been clarified yet. Further, this cell is not found in all gymnotiform genera, as shown by Quintana et al. (2011) for the genus *Brachyhypopomus*.

EOD physiology

Every EOD starts in the pacemaker, where neurons with an intrinsic periodical activity (pacemaker cells), functioning synchronously by means of electrical and chemical synapses, send a command to the electrical organ. This signal is first delivered to the relay cells, still in the pacemaker nucleus, which send their axons to contact electromotive neurons of the spinal fibers that innervate the cells of the EO. Thicker nerve fibers tend to enervate the more caudal sectors of the electrical organs, so that the pacemaker signal tends to arrive simultaneously along the entire EO. This is particularly relevant for fish that may attain large sizes such as the electric eel, *E. electricus*, which, according to local reports in South America, may reach more than two meters in length.

When the pacemaker command signal reaches the EO's electrocyte, acetylcholine is released into the synaptic cleft and, initially, sodium and potassium channels on the membrane are activated. In the electric eel, a resting electrolyte has a potential of about −90 mV (Bass, 1986; Keynes and Martins-Ferreira, 1953). When the ion channels open, electrical and chemical forces push positive ions into the electrolyte and negative ones out. With the depolarization, the interior of the electrolyte becomes positive, reaching a value of approximately +60 mV. As the internal potential of the electrocyte changes from −90 mV and starts to become positive, other voltage-gated ion channels may open and other ions, such as calcium, can contribute to the remainder of the action potential cycle. The process continues in a similar way as a muscle cell, where the ordered functioning of different types of ion channels causes the resting potential to be restored at the end of the EOD. A new cycle, or EOD, initiates with the next command started in the pacemaker.

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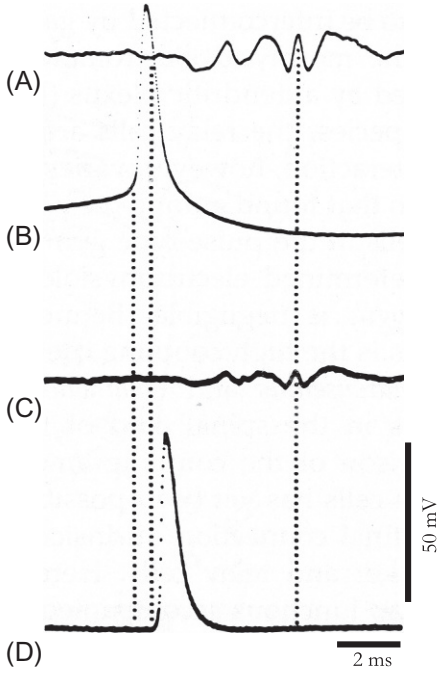

FIG. 6.5 Electrical activity in the electric organ and in the pacemaker nucleus of the gymnotiform genus *Gymnotus*. In (A) and (C), the command volley driving the electric organ is recorded in the spinal cord. In (B) and (D) is the intracellular recording of action potentials of pacemaker and relay cells, respectively. Both records are aligned with respect to the command volley marked by the rightmost dotted line. Note that pacemaker cells fire about 0.5 ms in advance of relay cells. *Adapted from Dye, J., and Meyer, J.H., 1986. Central control of the electric organ discharge in weakly electric fish. In: Bullock, T.H. and Heiligenberg, W. (Eds.), Electroreception. Wiley Series in Neurobiology. John Wiley & Sons, New York, pp. 71–102.*

It is standardized that the EOD of a given species corresponds to the signal picked up by a pair of electrodes placed in the water, close to the individual. In fish where the electrical organ is located along the body axis (the vast majority of cases), the positive electrode is placed in front of the head at a distance of about 50%–100% of the total length of the fish, with the negative electrode behind the tail at a similar distance. In electrogenic rays and uranoscopids, the electrical organ is in the dorso-ventral axis and, in this case, the electrodes are usually placed in the ventral and dorsal part of the individual, with the positive electrode on the dorsal side. For differential recordings, a reference electrode is placed around the midpoint between the positive and negative electrodes. Once the electrodes are positioned, the signal can be amplified, digitized, recorded, and visualized in an oscilloscope. In this case, it will be possible to visualize a positive inflection (up) of the baseline whenever there is current flowing toward the positive electrode (from the tail to head or from ventral to dorsal). Negative (down) inflection of the baseline happens when there is current flux in the opposite direction. In other words, if the flow of electrons occurs toward the positive (recording) electrode, there is an upward inflection in the line. If the electron flow is in the direction of the negative electrode, there will be a downward inflection in the baseline.

Obviously, once the EOD is the sum of the discharges of all the electrocytes, the anatomy of the EO and the physiology of the different electrocytes as well as their respective locations in the body of the fish contribute to the great diversity of EODs found in nature. There are electrocytes that only have one of the membranes excitable while others can have more; in some species the innervation may be in the anterior or ventral face while in others, it is in the opposite membrane. There are species in which different parts of the EO have different patterns of innervation, or even species with more than one EO. Due to these possibilities, it is not a surprise that each species of electric fish has a differentiated EOD. This makes the EOD a great tool for several studies from taxonomy to behavior to sexual selection to speciation, among others.

The anatomical and physiological diversity of the different types of electrical organs is directly reflected in the variety and complexity of EODs. The simplest discharge that can exist is that generated by an EO with electrocytes innervated in a single face, which will be the only excitable membrane of the cell. In this case, there will be only one-way current and the discharge will have a single inflection that may be positive or negative, depending on whether the excitable membrane is posterior/dorsal or anterior/ventral. However, as a didactic aid, the EODs can be classified according to their main biophysical characteristics.

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EODs' classification

Electric fish EODs can be subdivided into several categories, depending on the temporal aspects of the discharges, the maximum voltage output, and the number of phases they have. From the temporal point of view, EODs can be classified as pulse type or wave type.

An EOD is said to be a pulse when the duration of the discharge is smaller than the interval between two consecutive discharges, whereas in a wave-type EOD, the discharge duration is greater or of the same size as the interval between two consecutive discharges (Fig. 6.6).

In relation to voltage output, there are fish with strong EODs whose discharges can reach tens or hundreds of volts/cm and weak discharges that reach, in most cases, less than a volt but can reach a few volts/cm. Strong EODs can be used for defense and attack, whereas weak discharges are employed fundamentally in active electrolocation and electrocommunication. The great majority of electrogenic fish produce weak discharges, whereas in the Neotropic, only the discharges of the electric eel (locally known as "puraque"), some Torpedo rays, and the stargazers can be classified as strong. The puraque is special because it is the most powerful discharge known, being able to reach more than 600 V. A discharge of this amplitude can stun or even paralyze large animals. In the Amazon, there are numerous reports of people who have received an electric eel discharge, and deaths are not uncommon. It is known that the discharge of an adult puraque can be fatal to humans because this species produces about 6000–7000 strong discharges before depleting its storage of acetylcholine, the transmitter that is released by the motor neurons in the electrocytes. The only other freshwater fish that generates strong electric discharges is the African catfish *Malapterurus electricus*, also known as the Nile's electric catfish. Malapterurus can generate discharges of the order of 300 V/cm. The electric shocks of the Nile's catfish were known and used therapeutically in ancient Egypt, where they were recorded in murals and drawings as an animal of divine powers (Wu, 1984); see review in Moller (1995).

Finally, another type of EOD classification can be done in the function of the number of phases present in the discharges of the different species. Technically, a phase is the period in which the current in the EO flows in a certain direction, either from the head to tail or from the tail to head. The simplest type of discharge is the one with a single phase, when only one membrane of the electrocyte is excitable (usually the posterior one) and there is always current in one direction only. This type of discharge is found in practically all electrogenic fish during the larval stage and remains like that in several species, also in the adult phase. The electric eel and all gymnotiforms in their early life stages have a monophasic EOD (Kirschbaum, 1983; Kirschbaum and Schwassmann, 2008; Schwassmann and Assunção, 1989). This also applies to the large majority of catfish studied so far (Baron et al., 1994a,b,c, 1996a, 2001, 2008; Baron and Ol'shansky, 2009) as well as the rays (Baron et al., 1994d; Baron and Mikhailenko, 1977; Belbenoit, 1979, 1986) and the stargazers (Bennett and Grundfest, 1961; Baron and Mikhailenko, 1976). When this is interpreted in an evolutionary context, single-phased EODs appear as the most parsimonious option to be the plesiomorphic condition in electrogenic fish. The emergence of multiphasic discharges must be associated with complex selective pressures that include the appearance of new species, the

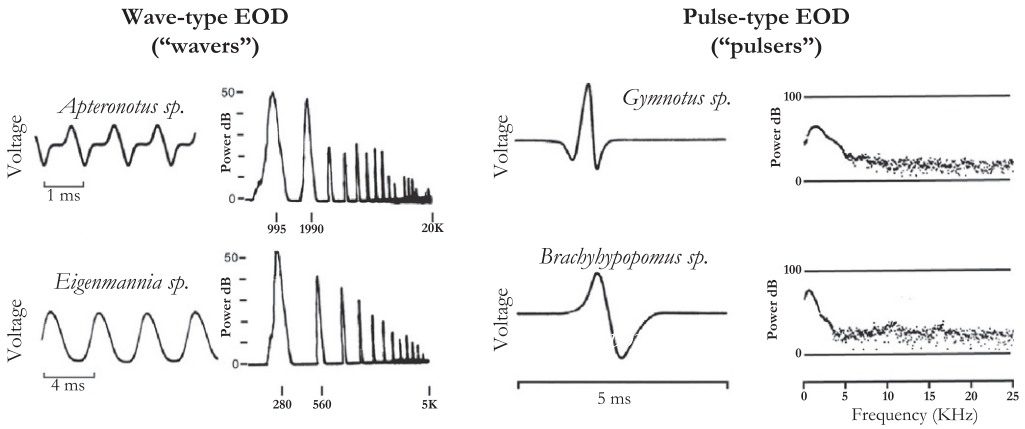

FIG. 6.6 Examples of wave- and pulse-type EOD in gymnotiforms. Wavers have an EOD in which the interval between two consecutive pulses either does not exist or is much shorter than the duration of the pulse. Within pulsers, the length of the pulse is much shorter than the interval. The waveform of each EOD is shown below the name of the genera and the respective power spectrum to the right of it. Note the time scales. Gymnotiform wavers may discharge in rates ranging from a few 10s of Hz up to more than 2300 Hz, whereas the pulsers' rate range between less than 1 Hz up to 150 Hz. *Adapted from Heiligenberg, W., 1977. Principles of Electrolocation and Jamming Avoidance Response in Electric Fish. A Neuroethological Approach. Springer-Verlag, Berlin, 85 pp.*

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adaptive reshaping of the EODs to become more effective for the EES efficiency in different types of habitats, and the avoidance of electroreceptive predators, among others (Alves-Gomes, 2001; Hopkins and Heiligenberg, 1978; Stoddard, 1999, 2002, 2006). The diversification of electrical signals and the consequent increase in the number of phases may have also been an effective way of consolidating the differentiation between populations and, therefore, speciation processes. Among the gymnotiforms, as an example of this diversity, there are species with up to eight distinct phases (Alves-Gomes, unpublished data). The specific relevance or putative advantage of a multiphasic EOD for communication or active electroreception is still to be revealed.

Electroreception in lampreys (order Petromyzontiformes)

Due to their anatomical characteristics, the electroreceptors of lampreys have long been identified as gustatory or chemical sensors in the form of buds ("taste buds"), as found in other fish, scattered throughout the epidermis. In the 1930s, it was observed that the synaptic ultrastructure of these organs resembled more prominently cells related to the octavolateralis system (Lane and Whitear, 1982), that is, specialized in the detection of mechanical stimuli. It was not until the 1980s (Bodznick and Northcutt, 1981; Ronan and Bodznick, 1986) that compelling physiological and anatomical evidence emerged showing that those organs observed in the lamprey epidermis were electroreceptors. The authors have termed them "end buds" by anatomically remembering the "taste buds" of other fish (Fig. 6.7A).

The lampreys' electroreceptors have a diameter between 25 and 65 μm, and are located on the surface of the epidermis, establishing contact with the external environment. Each electroreceptor organ consists of several supporting cells and 3–25 sensory cells that extend to the surface (Ronan, 1986). In the apical face of each sensory cell, there is a grouping of 80–90 microvilli, and a single nerve makes contact with local groups of electroreceptors (Ronan, 1986). The described anatomical features differ conspicuously from the ampullary electroreceptors found in cartilaginous fish, basal teleosts, or amphibians that have ampullary electroreception. Such a scenario calls for a closer look at the phylogenetic relationships in the base of vertebrates, specifically at the evolutionary relationships between lampreys, hagfish (which do not have electroreceptors), and other vertebrates. A series of studies based on molecular data notes that hagfish and lampreys are sister groups, forming a clade called Agnatha (Heimberg et al., 2010; Kuraku and Kuratani, 2006; Mallatt and Winchell, 2007; Osorio and Retaux, 2008; Yu et al., 2008). On the other hand, morphological studies indicate that lampreys form the sister group of the Gnathostomata (Khonsari et al., 2009; Løvtrup, 1977). The second alternative, also adopted in this book (see Chapter 1), reflects more parsimoniously the appearance of electroreceptors in vertebrates.

Despite the anatomical differences, from the physiological point of view, the lamprey's electroreceptors, similarly to the ampullary electroreceptors found in nonteleosts, are excited by cathodic stimuli and are also more sensitive to low-frequency stimuli (Bodznick and Preston, 1983; Muraveiko, 1984). This reinforces the hypothesis of a common origin (homology) for the electroreceptors among nonteleost fish.

In experiments where the activity of the electroreceptive afferents of the anterior lateral line nerve is registered, in adult specimens of the genus *Petromyzon* and *Ichthyomyzon*, it can be seen that, in the absence of any stimulus, the afferent fibers maintain a basal firing activity that changes as a function of the stimulus (Fig. 6.7B). For cathodic stimuli generated by

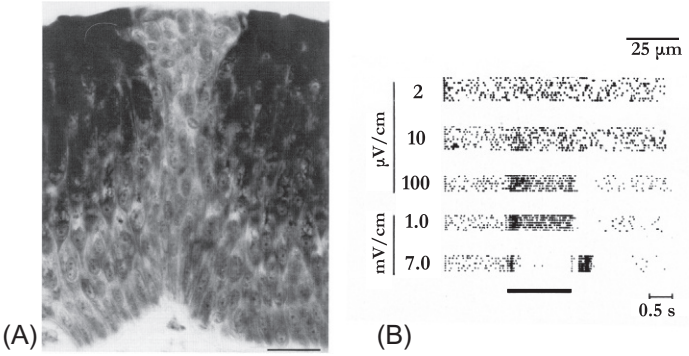

FIG. 6.7 Anatomy and functioning of an electroreceptor in lampreys. (A) Ampullary receptors in lampreys do not possess a canal connecting the lumen to the exterior. In lampreys, the electroreceptors are also called end buds and are immersed in the epidermis. (B) Physiological response of the electroreceptive fiber to a uniform field transversal to the subject. The positive pole is ipsilaterally placed in relation to the electroreceptor, that is, it is a cathodal stimulation. Each point in the raster represents a discharge recorded from the fiber. The stimulus presentation is denoted by the black bar in the bottom. For stimuli on the order of 2–10 μV/cm, there is almost no response. Stimuli between 100 μV/cm and 1 mV/cm induce a strong excitatory response. Strong stimulus cause the inhibition of the fiber's activity. *Adapted from Ronan, M., 1986. Electroreception in cyclostomes. In: Bullock, T.H. and Heiligenberg, W. (Eds.) Electroreception. Wiley Series in Neurobiology. John Wiley & Sons, New York, pp. 209–224.*

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uniform electric fields (direct current, DC), there is an increase in the number of discharges during stimulation, and the sensitivity threshold for this type of field was 1–10 μV/cm. This threshold increases exponentially for alternating fields above 1 Hz (Bodznick and Preston, 1983).

Despite the extensive literature on the morphological and anatomical aspects of lampreys' electroreceptors and respective brain structures, only a few studies are available so far about the behavior of lampreys in relation to electric fields. Chung-Davidson et al. (2004) measured the behavioral as well as brain responses of *Petromyzon marinus* in the parasitic stage to weakly electric fields ranging from 0.1–30 μV/cm. And Chung-Davidson et al. (2008) also measured the responses of adult lampreys to fields ranging from 0.1–10 V. The authors confirm the sensitivity of lampreys to weak electric fields and report a preference of the lampreys (adults and parasitic stage) to the cathode side of the tank. They also report differences in the effectiveness of weakly electric fields to induce swimming in the two ontogenetic stages. Differently form the parasitic form, adult lampreys are less responsive to stimulation with fields between 25 μV/cm and 1 mV/cm. The authors suggest that the electroreceptive system of lampreys may serve different purposes in the parasitic and adult stages, being more reproductive related in the latter.

Electroreceptors and EOs in cartilaginous fish (Class Chondrichthyes)

All the cartilaginous fish known (sharks, rays, and chimaeras) are electroreceptive and these fish have a single type of electroreceptor called Ampulla of Lorenzini. However, the definite demonstration that these specialized structures were actually electroreceptors took decades of study. Initially described by Lorenzini in the second half of the 17th century (Moller, 1995), the ampullae were initially interpreted as organs specialized in detecting pressure gradients. Subsequent laboratory experiments demonstrated that the ampulla also responded to temperature variations, and only in the early 1960s did Murray finally demonstrate the sensitivity of these organs to electric fields (Murray, 1960). Later, a series of studies including behavioral experiments consolidated the hypothesis that the Lorenzini ampullae were electroreceptor organs (Dijkgraaf, 1964; Kalmijn, 1966, 1971).

In relation to EOs, among all known species of cartilaginous fish, only a few rays of the genera *Torpedo*, *Narcine,* and *Raja* have them (Baron et al., 1994d; Belbenoit, 1979; Bennett, 1971a).

Electric rays were considered a unique example of fish that possessed electrical organs and electroreceptors, but did not have active electroreception. In addition to the problem caused by the high conductivity of salt water, which basically causes a short circuit in the EO and restricts the geometry of the EODs to a few millimeters around the body, the rays' ampullary receptors respond only to DC fields of low intensity, or alternating current (AC) fields with modulation rates varying from <0.1 to <15 Hz, but up to 50 Hz, depending on the species (Bodznick and Montgomery, 2005). As the discharges of the ray's EOs are pulse trains of relatively high frequency, the ray's electroreceptor organs would not have the capability to detect the fields generated by the individuals themselves (Baron and Mikhailenko, 1977; Belbenoit, 1970, 1979; Bodznick and Boord, 1986; Mortenso and Whitaker, 1973). In other words, electrogenic rays would not be able to detect the information originated by the distortions or interference in the electric field generated by their own electrical organs. However, behavioral experiments have shown that rays can use their discharges for communication during social interactions through active electroreception (Sisneros and Tricas, 2002; Tricas, 1980; Tricas et al., 1995). It is still uncertain whether all genera and species of electric rays use active electroreception, but given the sensitivity of the electroreceptors, it is quite possible that all the electric rays make use of the active electroreception for some type of behavior. On the other hand, it is well known that strong electrogenic rays use their electric discharges to stun and paralyze their prey (Belbenoit, 1986; Jacobsen and Bennett, 2013; Lowe et al., 1984).

Anatomy and physiology of ampullary electroreceptors in cartilaginous fish

Although all the cartilaginous fish are known for having ampullary electroreceptors, there are anatomical differences between the ampulla of freshwater and salt water fish. Szabo et al. (1972) described the anatomy and physiology of ampullary organs of the freshwater stingray *Potamotrygon* sp. and called them "microampullary organs." This was because the canal of *Potamotrygon* ranges between 300 and 500 μm long, whereas in marine species it can reach 1/10 of the body length in sharks and 1/3 of the body length in rays. The shorter size of the canal in freshwater species finds explanation as we examine the physical properties of the water, the fish skin, and the internal conductivity of the fish in relation to the external medium. In freshwater environments, the internal conductivity of the fish is higher than the surrounding water and the skin also has a relatively high conductivity (Kalmijn, 1974; Szabo et al., 1972; Szamier and Bennett, 1980). Under these circumstances, the larger voltage drop in freshwater environments is found across the fish's skin, that is, there is no need for long canals in the ampulla. Despite the anatomical differences, the ampullary receptors of freshwater and salt water elasmobranchs are homologous, and show similar physiological properties (Szamier and Bennett, 1980). However,

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physiological experiments show that the sensitivity of ampullary receptors in freshwater species is close to those registered to freshwater teleosts (ca. 100 μV/cm) and orders of magnitude lower than marine cartilaginous fish, which may reach 0.1–0.01 μV/cm (Kalmijn, 1966, 1974; Szabo et al., 1972). Further, studies with euryhaline species have shown that the same species, when moved from salt water to freshwater, have the sensitivity of their ampullary receptors reduced (Kalmijn, 1974; McGowan and Kajiura, 2009).

The theoretical, physiological, and behavioral bases elucidating the electroreception in cartilaginous fish were indubitably consolidated by a series of studies carried out by Adrianus Kalmijn, between the 1970s and 1990s. Kalmijn not only developed in detail the theory, but also demonstrated, through a series of behavioral experiments with rays and sharks, that marine cartilaginous fish have electroreceptors with a threshold of 0.01 μV/cm, so sensitive that this could allow these organisms to orient themselves in the electromagnetic field of the planet (Kalmijn, 1966, 1971, 1974, 1982, 1988a,b, 1997). According to the author, a shark or ray swimming at speeds of 50 centimeters per second (cm/s) induces electrical gradients between 0.1 and 0.2 μV/cm, values 10–20 times greater than the physiological threshold of the electroreceptors of these fish (Kalmijn, 1988a). The behavior of rays to laboratory-induced reversals of the Earth's magnetic field changed in a statistically significant way to fields on the order of 5 nV/cm. The results were no longer significant when field strength values were reduced to 2.5 nV/cm (Kalmijn, 1982). With this physiological threshold, elasmobranchs can use their electrosensory system not only for navigation using the magnetic field of the Earth as a compass for long and medium distances (Fig. 6.8), but also to orient themselves as a function of the geomorphological features of the marine ambient. Discontinuities in the chemical composition between rocks, sand, and water layers may generate electrical gradients. Therefore, in theory, the cartilaginous fish could distinguish geographic features on the seabed as valleys, caves, and other substrates, although no specific experiments have tested this hypothesis.

The ampullary receptors of cartilaginous fish follow the general physiology of a nonteleost ampullary receptor. The afferent nerves have a basal rate of discharge that can be accelerated or decelerated depending upon the polarity of the stimulus in the ampulla of the electroreceptor. Cathodic stimuli are excitatory, that is, when the interior of the ampulla becomes negative in relation to the basal surface of the electrosensitive cell, there is an acceleration of the nervous activity; when the interior of the ampoule is positive, there is an inhibitory action, with a decrease in the discharge rate (Fig. 6.9). The discharge rate is linearly proportional to the intensity of the stimulus for values ranging from −10 to +10 μV (Bodznick and Montgomery, 2005). It is also known that ampullary receptors of marine species are about 10 times more sensitive than receptors of freshwater species (Kalmijn, 1974; McGowan and Kajiura, 2009).

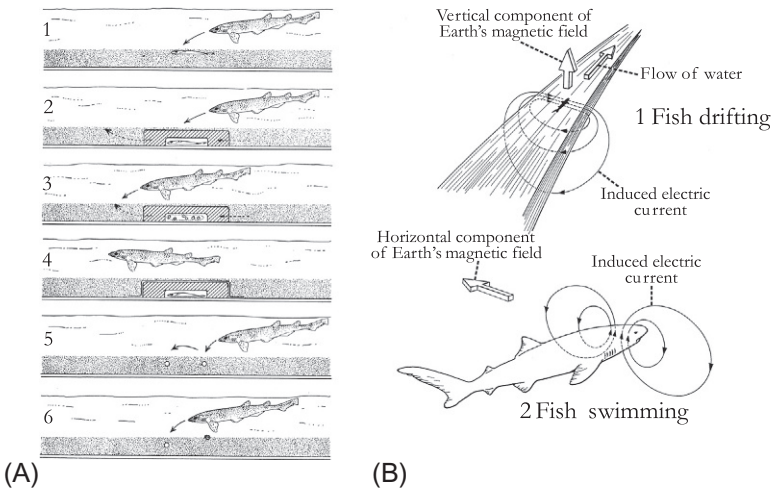

FIG. 6.8 Passive electroreception in cartilaginous fish. Theoretical and experimental studies have demonstrated that cartilaginous fish possess ampullary receptors sensitive enough to allow them to detect pray and orient themselves in relation to the Earth's magnetic field. (A) Behavioral (feeding) response of the shark *Scyliorhinus canicula* to chemical and bioelectrical fields. (A1) The flatfish (*Pleuronectes platessa*) is found by the shark when buried in the sand; (A2) when the flatfish is enclosed by an electrically transparent agar chamber, the shark still strikes at the correct position (solid line with *arrow*). The *dotted lines* indicate the flow of seawater through the chamber; (A3) when the live fish is replaced by fish flesh, the shark strikes where the chemical stimulus is stronger; (A4) if the agar chamber containing the flatfish is covered by an electrically insulating plastic film, the shark does not strike; (A5) electrodes simulating the electric field of a flatfish are attacked; (A6) in the presence of fish flesh (chemical and visual stimulus) and an electrical bait, the shark still strikes at the electrodes. (B) A cartilaginous fish either drifting (B1) or swimming (B2) in the ocean, induces electric currents in the electroreceptors. As a function of the fish's angle in relation to the horizontal and vertical components of the magnetic field, different amplitudes will result. Under these principles, cartilaginous fish can navigate in the oceans using the Earth's magnetic field as a compass. *Adapted from Kalmijn, A.J., 1974. The detection of electric fields from inanimate and animate sources other than electric organs. In: Fessard, A. (Ed.), Handbook of Sensory Physiology, vol. 3. Springer-Verlag, New York, pp. 147–200.*

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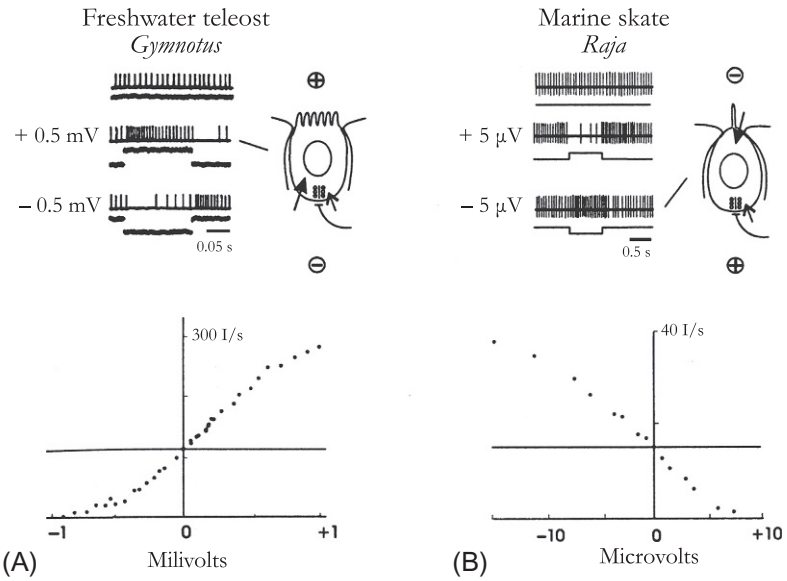

FIG. 6.9 Comparative response of low-frequency (ampullary) electroreceptors in teleosts and cartilaginous fish. In the upper trace of each schematic is the receptor's activity in the absence of stimulus. (A) Teleost's ampullary receptors increase their activity under anodal stimulation, that is, when the interior of the ampulla become positive and are inhibited under cathodal stimulation. With positive stimulus, the basal membrane of the receptor cell depolarizes, driving current into the cell (*arrows*). In the lower plot is the intensity of response of the afferent nerve as a function of the stimulus intensity. (B) In cartilaginous fish, the ampullary receptors are excited by cathodal (the interior of the ampulla becomes negative) and inhibited by anodal stimuli. When stimulated, there is a depolarization of the apical membrane of the receptor cell (larger *arrow* in B), which in turn depolarizes the basal membrane (*small arrow*), eventually releasing the neurotransmitter. In the lower plot, the intensity of response of the afferent nerve as a function of the stimulus intensity. Note the different scales for time and voltage in A and B and the greater sensitivity of receptors in the marine skate. *Adapted from Bodznick, D., and Montgomery, J.C., 2005. The physiology of low-frequency electrosensory systems. In: Bullock, T.H., Hopkins, C.D., Popperand, A.N., Fay, R.R. (Eds.), Electroreception. Springer Handbook of Auditory Physiology. Springer, New York, pp. 132–153.*

Additional behavioral experiments demonstrated the responses of rays and sharks for stimuli on the order of 0.1 μV/cm. For fields of this intensity, sharks respond by closing the ocular membrane while rays change the rhythm of respiratory movements. Stronger stimuli, on the order of 1–10 μV/cm, produce escape movements in sharks (Kalmijn, 1974, 1982; Wilkens and Hofmann, 2005).

Anatomy and physiology of EOs in rays

Among cartilaginous fish, only the rays of the genera *Torpedo*, *Narcine,* and *Raja* have EOs, and they all occur in the costal waters of the Neotropic. The EO of *Torpedo* consists of about 500–1000 parallel columns, each containing about 1000 disk-shaped electrocytes (10–30 μm thick and 5–7 mm in diameter in a 1 m wide fish) oriented transversely to the body plane (Bass, 1986). Both the ventral and dorsal surface of the electrolyte have a smooth appearance, despite having invaginations. The innervation of the organ in *Torpedo* is on the ventral surface and the EOD may reach up to 60 V (Bennett, 1971a).

In *Narcine*, there are two electrical organs: the main organ, with about 400 columns of 300–700 cells (7 μm thick, 2 mm in diameter in a 30 cm fish), innervated on the ventral surface while the accessory organ consists of about 10 columns of 200 cells each, innervated on the dorsal surface. The cellular morphology of the two organs of *Narcine* is similar to that of *Torpedo* and also like *Torpedo*, *Narcine* EOD may reach 60V (Macesic and Kajiura, 2009).

In *Raja*, the EO develops from hypoaxial muscle fibers in the tail and is located longitudinally along the tail of the adult individual. At about 10 weeks of life, the EO is easily identified and is formed by disc-shaped cells, similar to other electrogenic rays. The discharge of *Raja* is not considered strong and these fish, when isolated, rarely discharge spontaneously. Usually the EOD consists of a single pulse, or 1–2 pulses per second, and the voltage in the water is <1 V (Moller, 1995).

The basic physiology of these electrical organs is similar to the EO of other teleosts, that is, acetylcholine is released into the synaptic fossa, the electrocytes are depolarized, and the EOD is generated. The temporal pattern of discharges varies in different species, but the pulse is generally single-phased.

Differently from electrogenic teleost where the command nucleus (pacemaker nucleus) is well defined anatomically and physiologically, the command nucleus in rays is not known as well. Koester (2003) shows compelling evidence that the neurons responsible for the EO firing are located in the nucleus raphe magnum, also located, interestingly, in the ventral medulla as in the electrogenic teleosts.

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Electroreception in lungfish, *Lepidosiren* (order Lepidosireniformes)

There are three living genera of lungfish on the planet. Each genus is restricted to a different continent: the monotypic *Neoceratodus forsteri* lives in Australia; *Protopterus*, with four species, inhabits Africa; and *Lepidosiren paradoxa*, also monotypic, lives in the Neotropic.

Among all the fish groups discussed in this chapter, the three existing species of lungfish probably are the least known in terms of their electroreceptive capability. There are few studies that address the anatomy, physiology, or behavioral functionality of ampullary electroreceptors in this group, especially regarding the South American lungfish. According to Watt et al. (1999), electroreceptors in lungfish were originally described by Farenholz in 1929 as glands, and not cells specialized in detecting electrical gradients (Watt et al., 1999). Only in the 1970s did Roth demonstrate that *Lepidosiren* reacted behaviorally to low-intensity electric fields on the order of 0.3 $\mu V/cm^2$ (Roth, 1973). These fields are equivalent, in intensity, to fields generated during the respiratory movements of a fish at a distance of 5 cm. Because of this, Roth hypothesized that *Lepidosiren* could definitely detect the bioelectrical signals of a fish around 5 cm apart. Later, Watt et al. (1999) demonstrated, by behavioral experiments, that the Australian lungfish uses electroreception to locate weak electric fields nearby that are generated by living organisms.

The lungfish electroreceptors are concentrated in the head, where more than 2000 organs can be found (Northcutt, 1986). In *Lepidosiren*, the electroreceptors occur scattered over the trunk, reaching up to the caudal fin (Fig. 6.10). In the body and tail, these organs are less dense and occur in groups of three to five. Although the number of electroreceptors has not been counted in the trunk, it is estimated that there should be a few thousand in this region (Northcutt, 1986). The lungfish's receptors are immersed in a thick epidermis and have a form of ampule or pear, with an opening to the external environment. At the base of the ampulla, the sensory cell has a club-shaped structure, or a primitive kinocilium and some microvilli (Roth and Tscharntke, 1976; Watt et al., 1999). As in other electroreceptive fish, the fibers that innervate the receptors have tonic activity, that is, they are constantly active, producing spikes; modulations of the external electric field modify the activity pattern of the fibers (Roth, 1973), as can be seen in Fig. 6.10D for *Lepidosiren*. As in other nonteleost fish, *Lepidosiren*'s electroreceptors are stimulated by cathodic stimuli, that is, when the interior of the ampulla is positive and there is an electrochemical force "pushing" calcium ions inward into the apical membrane of the receptor cells. They differ from teleosts because, in the latter, the excitation is due to an anodic stimulus. In this case, the interior of the ampulla becomes negative and there is an electrochemical force "pulling" calcium ions through the basal membrane of the electroreceptor. As mentioned anteriorly, this physiological difference corroborates the nonhomologous condition of the teleost and nonteleost ampullary electroreceptors.

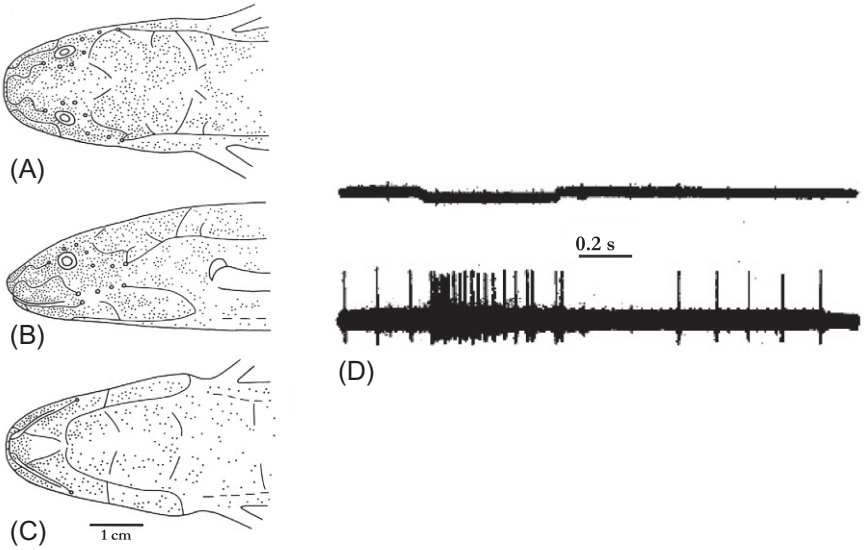

FIG. 6.10 Distribution of electroreceptors in the lungfish. In the dorsal (A), lateral (B), and ventra (C) views, it is possible to observe the location (*black dots*) of ampullary receptors in the Australian lungfish (*Protopterus*). Open circles represent pores of the lateral line and the solid lines are lateral line grooves. In (D), we see the physiological response of an electroreceptor organ in *Lepidosiren* (the South American lungfish). In the upper trace, we see a cathodal stimulus with a DC electric field near the body surface. In the lower trace, we see the firing of an afferent fiber innervating the electroreceptors. *Adapted from Northcutt, R.G. 1986. Electroreception in nonteleost bony fish. In: Bullock, T.H. and Heiligenberg, W. (Eds.), Electroreception. Wiley Series in Neurobiology. John Wiley & Sons, New York, pp. 257-285; and Roth, A. 1973. Electroreceptors in brachiopterygii and dipnoi. Naturwissenschaften 60, 106–106.*

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Electroreception and EOs in catfish (order Siluriformes)

At the beginning of the 20th century, Herrick (1901) anatomically described a sensory organ in a species of North American ictalurid catfish, which he called "small pit organs" (Herrick, 1901). At the time, the author had no idea about the function of these cutaneous organs, but noted their anatomical difference in relation to the taste buds that catfish also have scattered throughout the epidermis. In 1917, Parker and Van Heusen made the first behavioral observations, also with a North American catfish (genus *Ameiurus* when the work was published, but now reclassified as *Ictalurus*), and showed from a behavioral point of view that these catfish reacted differently when stimulated by metallic and nonmetallic objects. Although the authors had shown that catfish were responding to the difference in electrochemical potential, they associated this response with direct stimulation of the taste buds. Approximately 15 years later, Japanese researchers (Hatai and Abe, 1932; Hatai et al., 1932) reported changes in the behavior of catfish of the genus *Parasilurus* in the hours before earthquakes and associated these changes in behavior with changes in the earth's electromagnetic field during these extreme geological events. These studies supported an attempt to establish an earthquake warning system by having catfish with biosensors. Also in the 1930s, the sensitivity of catfish to galvanic stimuli (Kokubo, 1934) and even electric currents (Uzuka, 1934) was also confirmed. However, it was only in the 1950s that small pit organs were anatomically classified as specialized electroreceptor organs (Lissmann, 1958). The hypothesis that catfish would be electroreceptive was later confirmed by the studies of Dijkgraaf (1968), when the author noted that the electroreceptive sensitivity was lost with the ablation of the nerves of the lateral line system but not with those of the gustatory system. Finally, still in the late 1960s, Roth demonstrated directly through electrophysiological tests that the sensory organs previously described were sensitive to electrical fields (Roth, 1968). Further work has shown that electroreceptive capacity is present in all living catfish, as electroreceptors and specialized brain nuclei have been found in all catfish studied so far (Bullock, 1979; Kalmijn, 1974). This fact is particularly important from an evolutionary point of view because it is known that catfish and electric fish are sister groups; therefore, it is assumed that the common ancestor of these two fish lineages was already electroreceptive (Alves-Gomes, 1995, 2001, 2010; Betancur-R et al., 2017; Fink and Fink, 1981, 1996; Hughes et al., 2018).

Anatomy and physiology of electroreceptors in catfish

In catfish, all the electroreceptors described so far are of the ampullary type, with the exception of a single species of Neotropical catfish of the genus *Pseudocetopsis* that, according to the authors, has ampullary as well as tuberous receptors (Andres et al., 1988). Unfortunately, no further studies on the physiology or behavioral functionality of these tuberous electroreceptors were pursued in *Pseudocetopsis* and their ethological importance as well as the behavioral habits of this genus remain to be studied.

The ampullary receptors can be found in siluriforms over the entire body surface, but are absent in the barbels. The highest densities of the receptors are located in the dorsal region of the head and in the tail region, whereas the lowest densities are associated with places where the fish's own bioelectrical fields are stronger, such as in the mouth region, opercular slit, and the anus (Peters et al., 1974).

Catfish electroreceptor cells transmit information to the CNS via bipolar ganglion cells of the anterior and posterior branches of the lateral line nerves (Finger, 1986). These bipolar neurons emit processes in opposite directions in the direction of the periphery where they innervate the ampullary organs and, toward the CNS, terminate in the rhombocephalic region (Finger, 1986). Despite the mixture of sensory information on the lateral line nerves arriving from both the lateral line sensors and the electroreceptors, the information is segregated in the primary sensory nucleus of the cerebral bulb, with each type of afferent (mechanoreceptor or electroreceptor) ending in separate locations. A detailed description of the anatomy associated with the catfish electrosensory system is available in Tong and Finger (1983), McCormick (1983), and Finger (1986).

Catfish ampullary electroreceptors have a relatively low sensitivity threshold, although there is a wide range of thresholds reported in the literature, especially because these measures can vary greatly depending on the experimental conditions, water conductivity, and the initial state of the animal, among other variables (Finger, 1986). In general, similarly to what has been reported of organisms having representatives in freshwater as well as in marine environments, the electroreceptors of marine catfish are more sensitive than freshwater species. For instance, in the marine genus *Plotosus*, behavioral responses were elicited with stimuli current densities on the order of nA/cm^2 for voltage gradients of 0.5 μV/cm, whereas for freshwater species such as *Parasilurus* sp., the threshold is on the order of mV/cm for current densities between 0.1 and 5.5 $\mu A/cm^2$ (Kalmijn, 1974). In *Kryptopterus* (freshwater catfish), physiological responses in the afferent fibers of the electroreceptors were elicited with a stimulus of 0.1 mV/cm when applied at the ampullary opening (Teeter and Bennett, 1981). In other studies, authors (Kalmijn, 1974; Peters and van Wijland, 1974) determined that the behavioral threshold for

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the freshwater catfish *Ictalurus* is 1 μV/cm in fields with current densities of 0.5 nA/cm^2. These values were established in behavioral experiments and therefore are called behavior thresholds. The physiological thresholds measured directly in the electroreceptors are larger, on the order of 3.0 nA/cm^2 in fields of 6 μV/cm (Finger, 1986; Kalmijn, 1974). Behavioral thresholds tend to be lower than physiological thresholds due to a series of filtering mechanisms and noise cancellation at different levels of the CNS (Bodznick and Montgomery, 2005).

In relation to the tuberous electroreceptors discovered in *Pseudocetopsis* (Andres et al., 1988), there are still no new data available to subsidize any further discussion about their thresholds, operating patterns, or behavioral functionality. Therefore, there is no certainty that these organs are really sensitive and respond to electric fields, although the anatomy of it, according to Andres et al. (1988), resembles the tuberous receptor of the African mormyriform species *Gnathonemus petersii*. Indirectly, it can be interpreted that the organ is also morphologically similar to the remaining mormyriforms as well as the South American gymnotiforms. It would be interesting to determine, for example, whether *Pseudocetopsis* can detect gymnotiform's EODs as they may occur in sympatry, or how these electroreceptors could confer some sort of adaptive advantage for these fish. On the other hand, the family Cetopsidae would be a good candidate to have members with electrogenic tissues in the Neotropic.

Anatomy and physiology of EOs in catfish

Interestingly, although the Neotropic contains the largest diversity of catfish among all the Continents, all six genera of electrogenic catfish described so far (*Malapterurus*, *Ompok*, *Auchenoglanis*, *Heterobranchus*, *Synodontis,* and *Clarias*) are restricted to Africa (Baron et al., 1994c, 1996a,b, 2008; Hagedorn et al., 1990; Morshnev and Ol'shansky, 1997). Baron and Ol'shansky (2009) also described electric discharges in *Parasilurus asotus* in agonistic interactions, the same catfish genus used by Hatai and Abe in 1932 in studies about catfish response to earthquakes (Hatai and Abe, 1932; Asano and Hanyu, 1987). This species inhabits Southeast Asia and Russia and is very popular in Japan for fish farming and sport fishing.

The multiple emergence of EOs in several African catfish and the Asian *Parasilurus* (Alves-Gomes, 2001) is, by itself, a fantastic biological model for studies on comparative and convergent evolution. Yet, there is no record of an electrogenic catfish in the Neotropic until now. Therefore, in this chapter, the details about the anatomy and functioning of the different EOs in this group will not be further addressed. For further details on the morphology, physiology, and behavioral functionality of EODs in catfish, the reader can refer to the literature cited in this chapter and more specifically the publications of Baron et al. (1994a,b, 2008), Bennett (1971a), Bennett et al. (1958), Moller (1995), Morshnev and Baron (2001), and Peters et al. (2002).

It should be noted, however, that the work of Peters et al. (2002) indicates that the bioelectrical fields, generated only by the muscular activity and permeable membranes of the North American *Ictalurus* catfish in the laboratory, can reach values of 50–100 μV/cm, which is above the physiological threshold of the electroreceptors of catfish. With this, the authors suggest that, even without EOs, these catfish could use the natural fluctuations of their bioelectric fields as a simple form of communication. If this is confirmed, there is a great chance that Neotropical catfish will also use this artifice as a possible form of social interaction. There are still enormous possibilities of exciting research on this line of approach with Neotropical catfish.

Electroreceptors and EOs in South American electric fish (order Gymnotiformes)

The gymnotiforms are, to date, the only freshwater fish of the Neotropic that, in addition to having electroreceptors, also have electrical organs. With the exception of the members of the family Apteronotidae, all other gymnotiforms have myogenic electrical organs, that is, organs embryologically derived from muscle tissues (Kirschbaum, 1977; Kirschbaum and Schwassmann, 2008). In the case of the apteronotids, the group possesses an electrical myogenic organ during the larval stage, but as the individuals grow, the myogenic organ degenerates and is functionally replaced by the neurons that originally innervated the larval organ (Kirschbaum, 1977). In this way, the synapses involved in the EOD of the apteronotids are all basically electrical and, consequently, faster than the chemical synapses. This allows these fish to discharge their electrical organs at rates that can reach more than 2500 Hz (Dye and Meyer, 1986).

There are two basic types of electroreceptors in gymnotiforms, both already addressed before: ampullary and tuberous. Although the teleost ampullary receptors are not homologous to the ampullary receptors found in cartilaginous fish and other nonteleost fish, their respective anatomy and physiology are strikingly similar. Gymnotiform's ampullary system detects DC (direct current) electric fields and low frequency AC (alternating current) fields, generally smaller and slower than 15 Hz. Tuberous receptors, in turn, are sensitive only to AC fields and the sensitivity peak of each species is adjusted to

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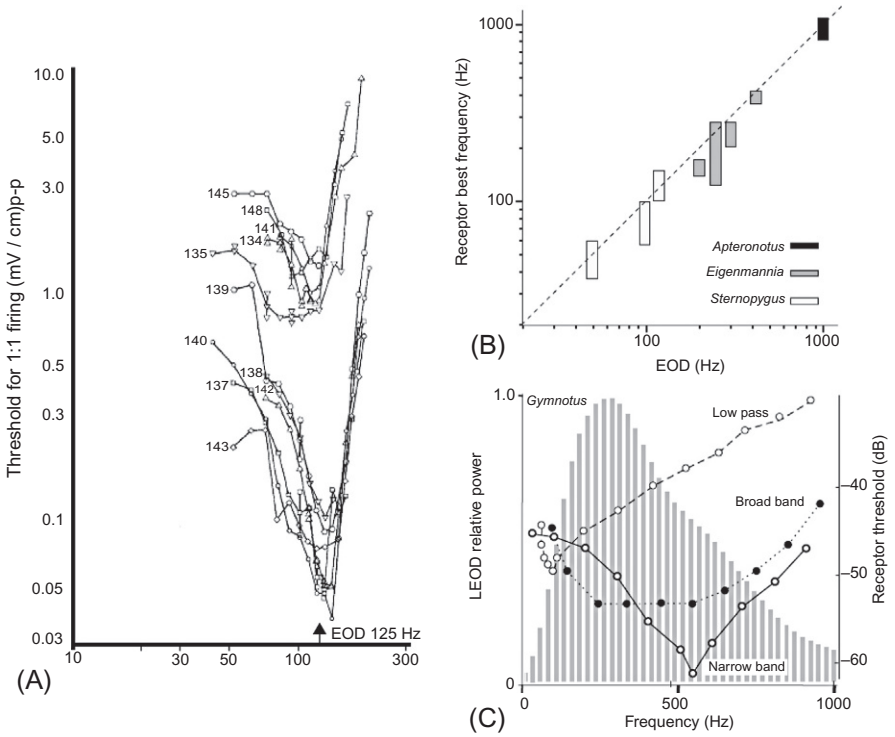

FIG. 6.11 Tuning curves for tuberous electroreceptors in gymnotiforms. (A) Recordings from several fibers (numbers) of a single specimen of *Sternopygus macrurus*. The upper cluster of traces corresponds to P-type tuberous organs, whereas the lower cluster, possessing a lower threshold, is T-type tuberous organs. Note the fine tuning of the electroreceptors around the discharge frequency of the fish (125 Hz). (B) Best response curve for electroreceptors of three wave-type gymnotiform species as a function of their own discharge rate. (C) Tuberous electroreceptors of the pulse-type gymnotiform genus Gymnotus, where three functional types of organs can be distinguished: the first works as a low-pass filter (*open circles*) best tuned to the lower frequencies; the second (*filled circles*) has a broad band of sensitivity covering almost the entire spectrum of the EOD; and the last one has its best response near the dominant frequencies present in the EOD's spectrum, represented by the gray bars. *Adapted from Hopkins, C.D. 1976. Stimulus filtering and electroreception—tuberous electroreceptors in 3 species of gymnotoid fish. J. Comp. Physiol. 111, 171-207; and Caputi, A.A. 2004. Contributions of electric fish to the understanding of sensory processing by reafferent systems. J. Physiol. Paris 98, 81–97.*

the EO's frequencies of the species considered (Fig. 6.11). That is, tuberous electroreceptors work by constantly monitoring the electric field generated by the individual's own EODs and their modulations. There are at least two basic types of tuberous electroreceptors in gymnotiforms (Carr and Maler, 1986; Zakon, 1986): one type detects the time in which each EOD occurs (called "time coding units"), and a second type encodes the amplitude of the signal ("amplitude coding"). These two types can be subdivided into other classes, as will be seen below.

Each electroreceptor organ is innervated by a single afferent nerve, which has its cell body in the anterior ganglion of the lateral line nerve. The electrosensory lateral line lobe (ELL) is a bilateral nucleus located in the medulla and the only termination of the electroreceptive primary afferents. In other words, the information coming from all the electroreceptors spread over the body surface of the fish, being it ampullary or tuberous, converges ipsilateraly to this cerebral nucleus (Carr and Maler, 1986). The ELL can be divided into four segments from the morphological and physiological perspective and, at this hierarchical level of the nervous system, the projections of the electroreceptors are already segregated because the information of the ampullary organs arrives only in the more medial segment while the terminations from the tuberous receptors reach the centromedial, centrolateral, and lateral segments of the ELL (Fig. 6.12A). In each of the segments, there is a somatotopic representation of the fish body, that is, the location of each electroreceptor in the fish's body is spatially maintained in each of the ELL segments (Fig. 6.12B), allowing the fish to receive and compare the information originated from electroreceptors on eight electrosensitive maps. From the ELL, the electrosensitive information is sent mainly to the nucleus praeeminentialis and to the torus semicircularis. The anatomy and physiology related to the processing of the electrosensory information in the CNS will not be addressed in this chapter, but Fig. 6.12 summarizes in a didactic way the work of several laboratories and publications that the reader can consult. These include Bastian (1986), Carr and Maler (1986), Carr et al. (1981, 1982), Heiligenberg (1977, 1987, 1988), Krahe and Maler (2014), Maler et al. (1982, 1991), Sas and Maler (1986), and Shumway (1989a,b).

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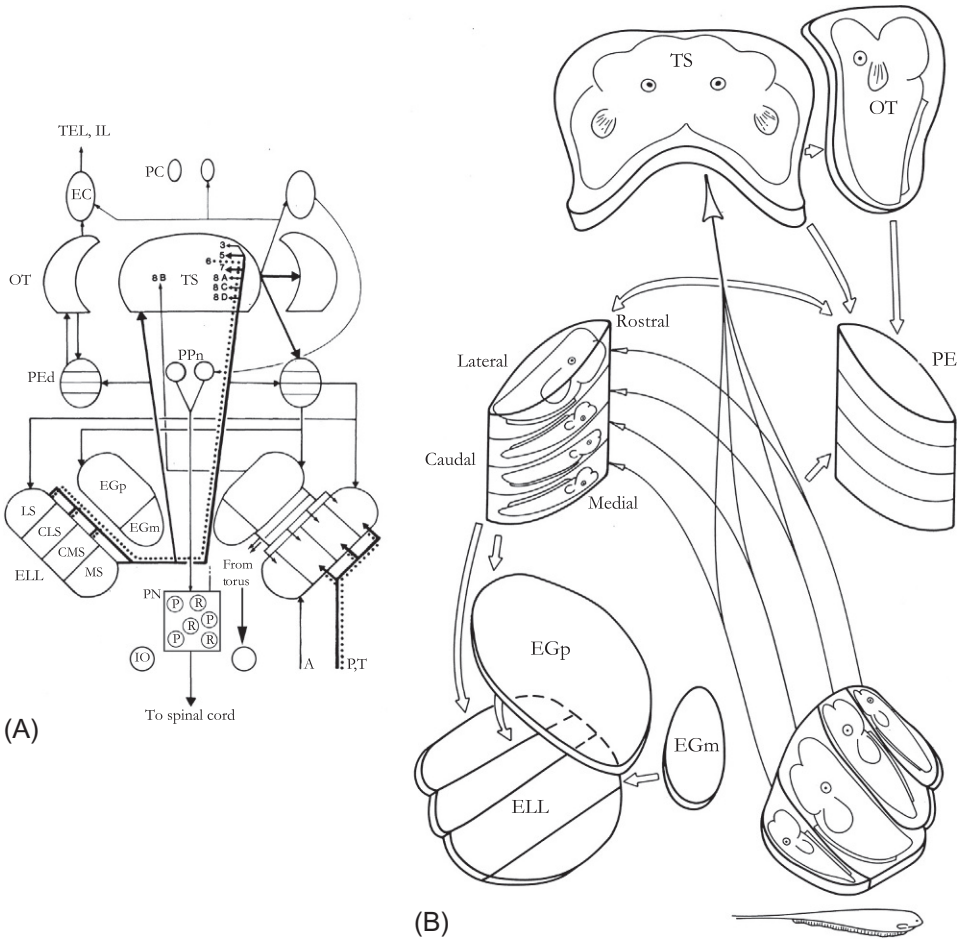

FIG. 6.12 Schematic representation of the connection of the electrogenic and electrosensory system (EES) system in gymnotiform fish, using *Eigenmannia* as an example. (A) Information arriving from the electroreceptive periphery through ampullary (A) and tuberous (subunits P and T) electroreceptors arrives at the electrosensory lateral line lobe (ELL) in a segregated fashion, with ampullary afferents connecting to the medial segment (MS) and tuberous afferents in the remaining segments: lateral (LS), centrolateral (CLS), and medial (MS). The ELL projects primarily to several layers of the torus semicircularis (TS) and to the dorsal portion of the preeminentialis nucleus (PEd). Both the TS and the PEd project to the optic tectum (OT). The TS also projects to the electrosensory nucleus (EC) and the later sends information to the prepacemaker nucleus (PPn), the first level of control of the electrogenic portion of the system. The PPn is the ultimate controller of the EOD firing rate in gymnotiforms, as the axons from this nucleus contact the pacemaker nucleus (PN), driving its rhythm up or down. The PN is formed by two types of cells: pacemaker (P) and relay (R) cells. The integration of this information at different levels of the cerebral hierarchy allow the electric fish to use the EES for electrolocation and communication. (B) The connections of the EEL in *Eigenmannia*. The EEL receives somatotopically ordered input from the electroreceptive periphery maintaining the corporal geometry clearly represented not only in the EEL, but also in several other nuclei of the EES, such as in the preeminentialis nucleus (PE), OT, and TS. For better comprehension, the connections are shown in only one side of the brain. Other abbreviations: *EGm*, Eminentia Granularis medialis; *EGp*, Eminentia Granularis posterior; *TL*, Telencephalon; *IL*, Inferior Lobe of the diencephalon; *IO*, inferior olivary nucleus; *PC*, posterior commissure. *Adapted from Carr, C., and Maler, L., 1986. Electroreception in gymnotiform fish. In: Bullock, T.H. and Heiligenberg, W., (Eds.). Electroreception. Wiley Series in Neurobiology. John Wiley and Sons, New York, pp. 319–373.*

Anatomy and physiology of ampullary electroreceptors in gymnotiforms

The ampullary organs of South American electric fish are very similar to catfish electroreceptors. In this case, however, the similarities are due to the homology, since gymnotiforms and catfish are sister groups (Alves-Gomes, 2001, 2010; Betancur-R et al., 2017; Fink and Fink, 1981, 1996; Hughes et al., 2018). Therefore, it can be assumed that the immediate common ancestor of both groups was already electroreceptive due to presence of ampullary electroreceptors, at least. Further, it is fair to estimate the time of existence of the common ancestor of catfish and electric fish, and consequently of ampullary electroreceptors within the Otophysi, at around the middle-upper Cretaceous (Alves-Gomes, 1999, 2010; Hughes et al., 2018).

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In general, in freshwater teleosts, the canal of the ampulla is shorter (100–200 μm in length) than in marine teleost fish, very much like the condition found in cartilaginous fish. However, differently from rays and sharks, where the ampullae can congregate in groups around a single canal, in the teleost each canal leads to a single ampulla (Zakon, 1986). The difference in canal size, as already described previously, is a consequence of the differences between the internal conductivity of the fish's body, the skin resistance, and the conductivity of the medium where they are found (Kalmijn, 1974).

These receivers of low-frequency electrical signals, in gymnotiforms, are scattered over the head and distributed in fixed paths over body surface. The wall of the ampulla has between three and six layers of flattened cells and is securely connected through desmosomes and tight junctions; these give them a high electrical resistance. Hence, the voltage gradient is directed more effectively to the sensory cell located at the base of the ampulla. Each ampulla has between two and eight sensory cells that slightly protrude toward the lumen (Fig. 6.4), exposing about 15%–20% of the sensory surface into the lumen (Jørgensen, 2005). Sensory cells have microvilli scattered on their apical surface (exposed to the lumen) about 1 μm in length. Each ampulla has a single nerve terminal with about 10–15 synaptic bodies, each about 5 μm in diameter. The single afferent fiber contacts all the sensory cells of an ampulla and can form a cluster with more than 20 arranged in the form of a "rosette" (Zakon, 1986). Each sensory cell has a diameter between 10 and 15 μm and contains a nucleus displaced to its apical portion. While the apical surface of the sensory cell contacts the lumen of the ampulla, its base is immersed in a layer of support cells, where there is contact with the afferent nerve fiber (Jørgensen, 2005; Zakon, 1986).

Like other ampullary receptors already described in this chapter, in the absence of any electrical stimulus, the afferent fibers that innervate the receptors have a constant and regular tonic activity of discharge that can increase or decrease depending on the polarity of the stimulus. Moreover, gymnotiforms' ampullary receptors accelerate their discharge rate when stimulated by anodal stimuli, and decrease its rate of discharge when the lumen of the ampulla becomes negative, i.e., under cathodic stimuli (Fig. 6.9). The response intensity of the electroreceptors has a quasilinear relationship with the amplitude of the stimulus, with stronger stimuli provoking stronger responses.

There are two basic and distinct ways of measuring the physiological threshold or sensitivity of electroreceptors: by recording the activity of the afferent fibers of the electroreceptors when a stimulus is presented (electrophysiological threshold), or by registering changes in some behavioral parameter of the studied fish (behavioral threshold) when an electric field is applied in its surrounding. Although one can assume that low-frequency stimuli will only excite the ampullary system, in behavioral experiments the contribution of the tuberous electroreceptors has not been quantified. It is estimated that the smallest potential difference that the ampullary electroreceptors of freshwater fish can detect is 10 μV/cm, which means a value between 10 and 100 times larger (less sensitive) than the values found for *Plotosus*, a genus of marine catfish (Kalmijn, 1988a). Although there are behavioral thresholds established for a few gymnotiform species, there are not specific studies measuring and comparing the sensitivity of ampullary receptors across species (Kalmijn, 1974; Knudsen, 1974; Peters et al., 2007). Peters et al. (2007) proposed 1 μV/cm as the average limit of detection of electric gradients by freshwater teleost's ampullary systems, and of 20 nV/cm for marine fish, after the interpolation of values for several species under both electrophysiological and behavioral studies. In their compilation, Peters et al. (2007) also registered the thresholds for three gymnotiforms, citing the studies of other authors. For *Gymnotus*, the electrophysiological threshold reported for Nobuo Suga in 1967 is of 58 μV/cm (5.8×10^{-5} V/cm), whereas for *Apteronotus albifrons* and *Eigenmannia virescens*, the behavioral threshold reported by the authors was of 0.6 μV/cm (6.0×10^{-7} V/cm) and 50 μV/cm (5.0×10^{-5} V/cm), respectively, as cited in Knudsen (1974). The study of Knudsen (1974) brings an interesting perspective into this matter. The author tested the behavioral thresholds for the two genera aforementioned with alternating electric fields of several frequencies. For frequencies below 20 Hz, that is, within the range of the ampullary receptors, the values are basically the same as those reported by Peters et al. (2007). Surprisingly, *Eigenmannia* appears to be near 100 times less sensitive than *Apteronotus*. However, when frequencies near the fish's own EOD rate are considered (around 400 Hz for *Eigenmannia* and 1000 Hz for *Apteronotus*), both thresholds dropped to very similar levels: 0.2 μV/cm for *Apteronotus* and <0.3 μV/cm for *Eigenmannia*. The sensitivity of the ampullary receptors of the remaining gymnotiforms should be between the values mentioned above, but specific studies still need to be done.

Anatomy and physiology of tuberous electroreceptors in gymnotiforms

Tuberous electroreceptors are physiologically tuned to detect the fish's own EODs and the gymnotiforms possess two basic types of tuberous electroreceptors: the first detects the timing of each discharge and a second type codifies the amplitude of the field generated during an EOD. These receptors are also able to capture the modulations in the field originated by different types of objects and organisms as well as by the EODs of other fish (Caputi, 2004; Von der Emde, 1999). Therefore, the tuberous system is fundamental during active electroreception and communication. The two types of tuberous organs in gymnotiforms can be subdivided into different subclasses, depending upon the type of EOD each fish possesses. Electric

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fish that have pulse-type EOD (pulsers) have tuberous receptors of subtypes M and D while the wave-type species (wavers) have T- and P-type electroreceptors (Carr and Maler, 1986). Some authors adopt the letter "B" for the type-D electroreceptor (Moller, 1995). The M-type (pulse markers) in pulse-type fish and the T-type (phase coders) in wavers have a homologous function and fire 1:1 with the EOD of the individual. Therefore, they precisely encode the moment of each individual discharge. However, they do not provide information about the amplitude of the signal. For this, there are the D-type (burst coders) in the pulsers and P-type ("probability coders") in the wavers.

In relation to the physiological sensitivity of tuberous electroreceptors, there is a great deal of variation between species and the curve between the sensitivity threshold and the stimulus frequency is not linear (Caputi, 2004; Hopkins, 1976). Caputi (2004) offers a didactic example of how different types of electroreceptors in *Gymnotus* are tuned to deliver their best response for different frequencies in a way that, together, they cover a large portion of the frequency spectrum of the species' EOD (Fig. 6.11).

In behavioral experiments, when the sensitivity of all electroreceptors is evaluated together, *Apteronotus* shows a bimodal curve in relation to the stimulus' frequency. The thresholds are of the order of 0.6–0.9 μV/cm for frequencies between 5 and 15 Hz; 0.9 μV/cm for frequencies of 200–300 Hz, and 0.2 μV/cm for frequencies between 700 and 2200 Hz (Knudsen, 1974). For *Eigenmannia*, another waver, the curve is unimodal with a threshold of 0.2–0.3 μV/cm and with a peak centered on the fundamental frequency of EOD (Knudsen, 1974; Zakon, 1986). From this data, it is clear that *Eigenmannia* is not as sensitive as *Apteronotus* to frequencies below 20 Hz.

The sensitivity of tuberous receptors also varies with the conductivity of the water. In *Apteronotus*, for example, the threshold for a 1000 Hz stimulus is <0.3 μV/cm in water with a resistivity of 0.5 kΩ × cm, and around 1 μV/cm when the water resistivity is 100 kΩ × cm. Further, for water with a resistivity of 1 kΩ × cm, the threshold for a 10 Hz sine wave stimulus is around 3 μV/cm; it is 0.7 μV/cm for a 200 Hz stimulus and <0.3 μV/cm for a 1000 Hz stimulus. In general, the fish is more sensitive to a frequency close to its own EOD fundamental frequency for water resistivity ranging between 0.5 and 100 kΩ × cm (Knudsen, 1974).

Figs. 6.11A and B summarize the basic information available for the response curve of tuberous electroreceptors for wave-type and pulse-type gymnotiforms, whose respective discharge rates range from about 50 Hz (in the "waver" *Sternopygus*) to about 2000 Hz (in the "waver" *Apteronotus*). It is assumed that in all other wave species, the best response frequency of the tuberous electroreceptors conforms to the carrier frequencies of their EODs. However, only a few species have been adequately studied.

In the case of fish with a pulse EOD, the available studies become scarcer. However, according to the published data and the theoretical understanding of the EESs, it can be expected that the tuberous electroreceptors of all the pulse gymnotiforms, like those of the wavers, are also physiologically tuned to the spectral characteristics of the EODs of each respective species. On the other hand, it is known that the spectral content of a pulse type discharge is quite different than the spectrum of a wave-type EOD. Basically, while the EODs of a waver fish have a peak of energy in the fundamental frequency and decreases in the higher harmonics, in pulsers the energy is not so concentrated around a single frequency. The frequency spectrum of a pulse-type EOD is broader (Fig. 6.6), with a significant amount of energy distributed over frequencies ranging from a few Hz to a few kHz. Given these biophysical characteristics of pulse EODs, it is expected that the tuberous electroreceptors of the pulse gymnotiforms also have a broader response curve, covering a higher content of frequencies present in EODs. Fig. 6.11C summarizes the response curve of the tuberous electroreceptors in the genus *Gymnotus*, a pulse-type fish. It can be noted that there are three physiologically different types of electroreceptors, working in tandem, nicely covering the full spectrum of frequencies present in the EOD. The first type has a sensitivity peak for the lower frequencies and is characterized as a low-pass filter; the second type responds basically in the same way for frequencies ranging from 200–600 Hz and is termed "broadband"; and the third has a higher, narrow sensitivity close to the dominant frequency of the spectrum and is called the "narrow band" (Caputi, 2004).

Anatomy and physiology of electrical organs in gymnotiforms

All gymnotiforms, during the larval stage, have a larval electrical organ derived from muscular tissue that may or may not become the adult's EO, depending on the species (Kirschbaum, 1977; Kirschbaum and Schwassmann, 2008). Studies on the ontogenetic development of the EO in this group recognize two types of primordial electrocytes: type A, in wave-type species; and type B, in pulse species (Kirschbaum and Schwassmann, 2008). Type B electrocytes apparently tend to differentiate directly into adult organs while the electrical organs of the wavers may or may not be replaced by another EO in the adult individual. The most extreme case is that of the family Apteronotidae, where the myogenic electrical organ degenerates, and a second electrical organ is formed purely by neurons (Kirschbaum, 1983).

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The EOD of each species is the product of the depolarization of all the electrocytes the species possesses. Electrocytes do not occur isolated, but rather grouped in specialized electrogenic tissues, the electric organs. The EOD waveform of a given species, captured under standardized arrangement of electrodes placed externally to the fish (and visualized on an oscilloscope screen), depends on several anatomical and physiological factors, among which the most relevant are: the number and location of the EOs a species has, the type of innervation (anterior or posterior) of the individual electrocytes, the presence of stalks in the electrocytes, the number of excitable membranes of each electrocyte, and the discharge synchronization of the different groups of electrocytes. Among the gymnotiform species, substantial differences in one or all of these items can exist, which creates a wide variety of EOD waveforms ranging from simple, single-phased (as found in species of the genera *Electrophorus*, *Gymnotus*, *Brachyhypopomus*, and *Microsternarchus*) up to eight phases, as in some species of the genus *Hypopygus* (Alves-Gomes, unpublished data). As for the duration, gymnotiform EODs can last from about 1 to over 15 ms (Alves-Gomes, unpublished data). However, this parameter is directly dependent on the arbitrary definition of where the EOD pulse starts and finishes. In general, the starting and ending point of an EOD is established as the point in which the baseline of the signal crosses a window of voltage around the baseline, defined as a percentage of the peak-to-peak voltage. For example, assume that an EOD has a peak-to-peak value of 1 V around 0, and the threshold chosen to establish the total duration of the EOD is 5% of the peak-to-peak voltage. Thus the length/duration of the EOD starts at the point in which the baseline crosses outward the value of 0.05 V, either toward the positive or negative values. Likewise, the end point is established when the baseline returns into the ± 0.05 V interval, toward the resting potential. In general, researchers use intervals between 1% and 5% of the peak-to-peak voltage in normalized EODs (Crampton and Hopkins, 2005; Hopkins, 1991; Stoddard et al., 1999). Obviously, the EOD duration is inversely proportional to the threshold used. Further, under special recording circumstances where the noise can be greatly diminished, the threshold can potentially be reduced to 0.1%–0.2%. Under these new thresholds, the EOD duration for the large majority of gymnotiforms sampled, especially the pulse species, is much larger, reaching in some species more than 20 ms (Alves-Gomes, unpublished data). It is still unclear how important these extensions of the pulses may be for active electrolocation or communication.

Among the gymnotiforms, there are three families of wave-type fish Sternopygidae, Eigenmannidae, and Apteronotidae) and four families of pulse fish (Hypopomidae, Rhamphichthyidae, Gymnotidae, and Electrophoridae), following the phylogenetic hypothesis proposed by Alves-Gomes et al. (1995). Interestingly, among the pulse fish, the tribe Microsternarchini (family Hypopomidae), which encompasses the genera *Microsternarchus*, *Procerusternarchus*, and *Racenisia* (Albert, 2001; Cox-Fernandes et al., 2014), represents an intriguing example of a homoplastic transition from pulse to wave-type EODs within the order because the large majority of the species within the Tribe have intervals smaller than the duration of the EOD (Alves-Gomes, unpublished data). Further, all the other genera within the Hypopomidae and their sister group (Rhamphichthyidae) are fish with a pulse EOD (Alves-Gomes et al., 1995; Albert, 2001; Sullivan, 1997). The EODs of microsternarchines are characterized by a pulse duration between 5 and 11 ms (measured with a threshold of 1% of peak-to-peak voltage), and are highly stable with coefficients of variation on the order of $<10^{-3}$ s for most species and reaching 10^{-4} s in *Procerusternarchus* (Alves-Gomes, unpublished data).

The anatomy of the electrocytes in gymnotiforms varies significantly depending on the species. For instance, in apteronotids, the electrocyte is the neuron itself, whereas in the myogenic organs the electrocyte morphology includes cylindrical (or cigar-shaped), square, or rectangular forms, with more or less invaginations (Caputi et al., 1994; Couceiro and Almeida, 1961; Kirschbaum and Schwassmann, 2008). Despite the different formats in their respective cells (Fig. 6.13), gymnotiform EOs usually are organized in the form of longitudinal, bilaterally paired structures that extend along the individuals' bodies. Depending on the species, these structures vary in number, ranging from less than five as in *Gymnotus*, up to tens, as in the case of the electric eel. The individual electrolytes are separated by layers of collagen (Couceiro and Almeida, 1961), and the discharge of each electrolyte is added to the composition of the full EOD. The electrolytes can be arranged in series, which contributes to the final EOD voltage, or in parallel, which contributes to the final EOD amperage. In *Electrophorus*, which is just longer than 1 meter in length, the discharge can reach more than 500 V and 1 A (Moller, 1995; Schoffeniels, 1961), but in nature, these fish can easily reach more than 2 m in length.

E. electricus, or the electric eel, is a special case within the gymnotiforms due to its various anatomical and physiological peculiarities. In relation to its electrogenic system, this fish has three different electrical organs: the main, the Hunter, and Sachs EOs. These three organs do not always discharge simultaneously. Depending upon which organs are recruited, there are two types of discharges: the first type is a relatively weak discharge, about 10 V of amplitude emitted at rates ranging from <1 per min up to 25 times per s. The Sachs organ produces this type of discharge during rest or navigation, functioning mainly for orientation. The second type is the strong discharge, which can reach more than 600 V and is produced when the main and the Hunter organ are also recruited, in addition to the Sachs organ. These discharges are generated by means of high-frequency peaks that arrive at up to 500 Hz (Bennett, 1971a; Moller, 1995) and are

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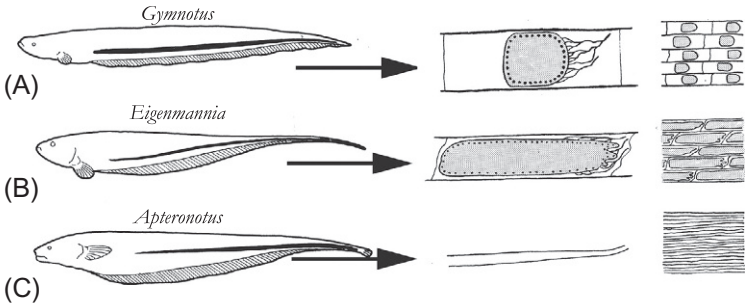

FIG. 6.13 Electrocyte anatomy in gymnotiforms. Schematic representation of a longitudinal section in three genera of South American electric fish, shown in the *left side*. Individual electrocytes are packed together in the electric organ (EO), as shown in the rightmost drawings. The electrocyte shape and innervation pattern vary among species. In (A) and (B), the electrocytes are innervated in the caudal membrane. Sometimes a given species may present electrocytes at different portions of the EO with distinct innervations patterns and number of excitable membranes (anterior and/or posterior). The sum of all electrocytes firing in synchrony generates the EOD of a particular individual. In the gymnotiform Family Apteronotidae (C), the muscle-derived cells that originated the adult electric organ degenerate and the EO are formed by the neurons that originally innervated the myogenic organ. Drawing not to scale. *Adapted from Couceiro, A. and Almeida, D.F. 1961. The electrogenic tissue of some gymnotidae. In: Chagas, C. and Paes de Carvalho, A. (Eds.) Bioeletrogenesis—A Comparative Survey of Its Mechanisms With Particular Emphasis On Electric Fish. Elsevier, Amsterdam, 3–13.*

used for defense as well as to paralyze and capture the prey (Catania, 2014, 2015). The electric eel discharges are monophasic and positive as a function of the innervation and excitability pattern of their electrocytes, which receive the terminals of the motor neurons on its posterior face, the only one electrically excitable. In this species, the EO becomes functional in individuals of about 15 mm in length (Schwassmann and Assunção, 1989).

Besides the electric eel, in the majority of the other gymnotiform species, there is customarily a single EO, but accessory organs are relatively common and have been described in several other genera of pulse species (*Brachyhypopomus*, *Hypopygus*, *Steatogenys*, *Rhamphichthys*, and *Gymnorhamphichthys*) and in a single genus of wave fish (*Adontosternarchus*) (Bennet, 1961, 1971a; Giora and Carvalho, 2018, Nijssen and Isbrucker, 1972, Sullivan, 1997). The presence of accessory organs causes EOD to become more complex from its spectral point of view and tends to increase the number of phases of the discharge. This, in turn, contributes to the large variety of EOD shapes and possibilities of interspecific distinction.

EOs in stargazers (Uranoscopidae)

Uranoscopids are also known as stargazers and electrical organs have been described, so far, for two genera: *Astroscopus* and *Uranoscopus* (Baron, 2009; Baron and Mikhailenko, 1976; Bennett and Grundfest, 1961; Dahlgren, 1906). According to phylogenetic studies based upon morphological characteristics, these two genera are not sister groups (Pietsch, 1989) and, in addition, there is evidence that in these two genera, the respective electrical organs are differentiated from different muscles. In *Astroscopus*, the EO derives from the ocular musculature (White, 1918) while in *Uranoscopus*, it is from sonic muscles (Mikhailenko, 1973), as reported by Moller (1995). Although the two EODs are monophasic, they differ in their respective orientations: in *Uranoscopus*, the current flow is in the head-tail direction, whereas in *Astroscopus* it is in the dorso-ventral sense (Baron, 2009; Bennett and Grundfest, 1961). This evidence, when combined, suggests that these organs may not be homologous. Further studies on the phylogenetic relationships of the eight genera within the family (Nelson, 2016) are fundamental for a better understanding of the evolutionary processes that culminated with EOs in this group.

The presence of EOs in uranoscopids becomes even more interesting as one considers the various peculiarities of the electrogenic system of this group. The basic function of these organs in the stargazers remains an unsolved question for different authors. The *Astroscopus* discharge is classified as intermediate between weak and strong because the potentials recorded in the air, for a fish of 50–75 cm, are of the order of 5 V (Baron and Mikhailenko, 1976; Bass, 1986; Bennett and Grundfest, 1961; Grundfest and Bennet, 1961). The potentials generated by *Uranoscopus* are of the order of 4.5 mV for a fish of 21.5 cm and with the electrodes positioned 20 cm away (Baron, 2009). In *Astroscopus*, the normal discharge pattern consists of a burst or pulse train that is followed, about 100 ms later, by a slower, discrete train of pulses that can last for several seconds. The bursts of pulses occur immediately before or during mouth opening to catch the prey, and the larger the prey size, the longer the duration of the discharge (Pickens and McFarland, 1964). However, electrical voltages of this magnitude, especially in conductive waters such as marine waters, are not enough to stun the types of prey normally captured by the uranoscopids, at least while the prey is out of the fish's mouth. The electric potential inside the fish's mouth has

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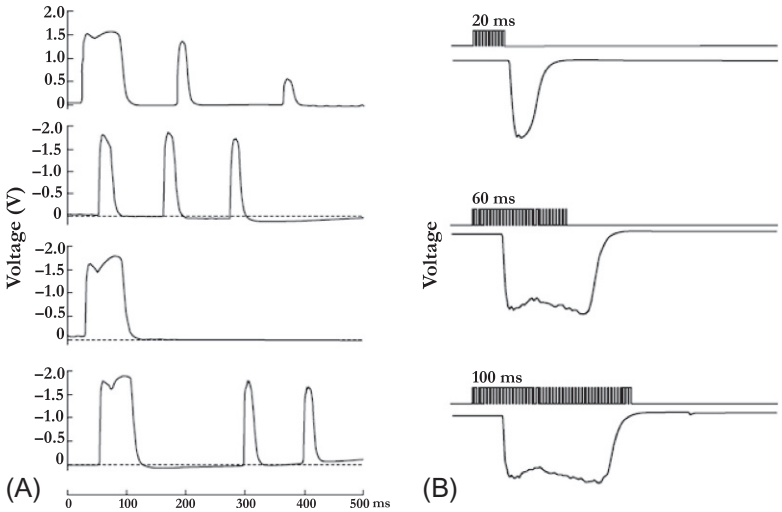

FIG. 6.14 Electric organ discharge in *Uranoscopus*. (A) Series of discharges from *Uranoscopus* obtained by mechanical stimulation. The pulse is monophasic, that is, there is current flow in only one direction. The positive and negative values obtained in the plots are dependent upon the position of the positive and negative electrodes in relation to the dorso-ventral axis of the body. (B) Electric organ discharges were also obtained by electric stimulation of several locations in the brain. The stimuli are represented by vertical bars on top of the EOD. Longer stimulations elicited longer discharges. Although EODs were produced when several portions of the brain were stimulated (midbrain, cerebellum, medulla, and spinal cord), the most consistent results and invariable latency of response were obtained when the stimulus was directed to the medial portion of the basal area of the fourth ventricle of the medulla oblongata. The medulla seems to be where the command center for electric organ activity is located in most of the electrogenic fish. *Adapted from Baron, V. D. 2009. Electric discharges of two species of stargazers from the South China Sea (Uranoscopidae, Perciformes). J. Ichthyol. 49, 1065–1072.*

not being measured but, according to Pickens and McFarland (1964), there is a correlation between the length of the prey and the number of pulses of the train.

In *Uranoscopus*, discharges can be triggered by the mechanical stimulation of the fish, when a train of pulses with up to six pulses per second is fired (Fig. 6.14). Each pulse can last between 30 and 100 ms. Another distinct aspect of this group, as we have seen previously, is the fact that uranoscopids do not possess electroreceptors at all, that is, they are not electroreceptive and the electrical discharges apparently are not part of any active electrolocation system nor do they have a communicative function. Apart from the possible use of the discharge to potentially inhibit or diminish prey movements within the mouth, which is debatable, there is not a robust and convincing evolutionary explanation for the presence and real utility of EOs in these fish. Baron (2009) suggests that the discharge of uranoscopids could also serve as a warning signal to electroreceptive predators. No anatomical details are available about the EO in *Uranoscopus*, but EODs have been physiologically registered for more than one species in the genus (Baron, 2009).

In *Astroscopus*, an EO can be found behind each eye, positioned dorsally in the head. The organ consists of approximately 150–200 layers of large and flattened cells (5 mm in diameter in a fish if 20 cm), forming a column that extends vertically from the dorsal surface of the head to the mouth palate (Bass, 1986; Bennett and Grundfest, 1961). Each layer is composed of about four large cells surrounded by about 10 smaller cells that overlap irregularly. The dorsal side of the electrolyte is smooth while the ventral face is full of invaginations that increase the surface area. The innervation of the electrodes in *Astroscopus* occurs by the smooth, dorsal face. Therefore, during the discharge, there is current going from the dorsal to the ventral side. This generates a single-phase negative EOD (if the positive electrode is placed above the fish's head).

The appearance of an EO in an organism that does not possess electroreceptive capability seems counterintuitive at first. On the other hand, the condition found in the uranoscopids could arguably be considered a very important example, from an evolutionary point of view, to question if nature always follows the most parsimonious path.

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Chapter 7

Color and physiology of pigmentation

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Introduction

By definition, color is the sensation that results from stimulation of the retina by light at certain wavelengths. A variety of color patterns are widely spread among animals (Bagnara and Hadley, 1973; Ligon and McCartney, 2016). When speaking of color, the inevitable inference is on what humans can see, or rather the wavelengths visible to human eyesight such as blue, yellow, red, etc. Nevertheless, the range of wavelengths visible to several groups of animals has proved to be more ample. For example, there are those capable of perceiving some that are completely invisible to humans, such as ultraviolet. Furthermore, photic conditions vary considerably in accordance with the prevailing environment and period of the year. Consequently, many animals, including fish, present a perception of color that is appropriate to specific survival strategies. This is the case with fish that live on the surface in light-exposed environments, and those that live underground. Likewise, besides the color within the environment itself, animals can also perceive those of other organisms (Aspengren et al., 2009).

The ability to present such a variety of colors presupposes the possession of molecules or structures that are capable of absorbing light at a certain wavelength, and emitting at a length complementary to that absorbed. In animals, the pigments responsible for color is contained in specialized cells, generically denominated chromatophores that, although mainly present in the skin, can also be encountered in other regions of the body, such as the abdominal cavity and around internal organs (Erickson, 1993; Hirata et al., 2005). In the vertebrates, these chromatophores originate from neural-crest cells, and in their differentiated state, possess dendrites that radiate from the cell body (Erickson, 1993) (Fig. 7.1). Pigments are stored in organelles, the so-called chromatossomes present in the cytoplasm of chromatophores. The vast majority of crustaceans, cephalopods, and ectothermic vertebrates present various types of chromatophores that are capable of adjusting the animal's color in distinct situations. On the other hand, birds and mammals present only one type of pigment cell, denominated melanocyte. The pigment produced, either melanin or pheomelanin, is transferred to the epidermic appendix. In addition, there is also the participation of the so-called structural colors, resulting from the interaction of light, as for example in the case of feathers.

Pigment cells, and in general all the pigment system present in animals, may be involved in various different functions. Even so, when considering color in teleost fish, protection and communication are apparently the most important (Bagnara and Hadley, 1973; Korzan et al., 2008; Ligon and McCartney, 2016).

The most common mechanism for color change in fish involves alterations in the intensity of the black, brown, or gray (melanistic colors) areas through the dispersion or aggregation of the pigment granules present in pigment cells. These

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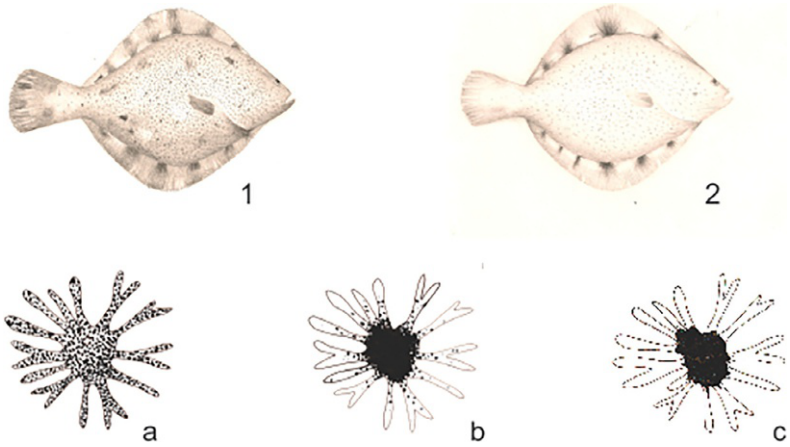

FIG. 7.1 Correlation between the distribution of pigments in melanophores and color. When the beads are dispersed (A) the animal darkens (1), and as they aggregate (B, C), the animal clears. *(Modified from Fujii, R., 1993. Cytophysiology of fish chromatophores. Int. Rev. Cytol. 143, 191–225. 1 and 2 original by P. R. Maciel Filho.)*

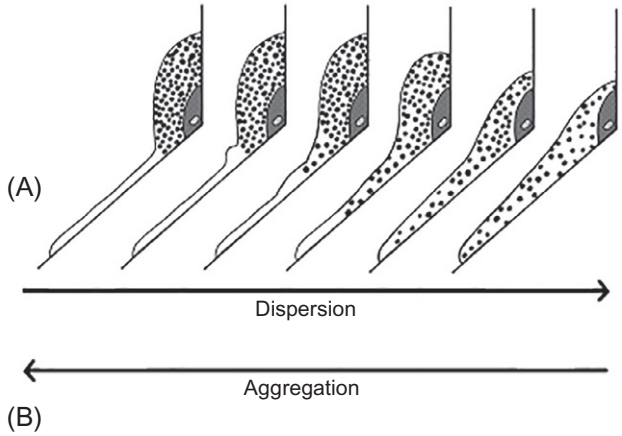

FIG. 7.2 Schematic representation of a chromatophore in different stages of pigment aggregation/dispersion. (A) Maximum aggregation, (B) maximum dispersion (original by Monique Rached).

alterations can occur in a matter of seconds. The behavior of pigment-containing cells is controlled by either the nervous or the hormonal systems, or by both together, with quicker or slower changes. Various hormones and neurotransmitters, such as the melanocyte stimulator hormone (MSH), the melanin concentrator hormone (MCH), and noradrenalin, are known for regulating chromatophore response by inducing either the perinuclear aggregation of pigment granules or their dispersion to dendritic projections (Fujii, 2000) (Fig. 7.2). Besides rapid or physiological response, these cells can also alter their number or size when the stimulus persists for a sufficiently long time. Alterations in color induced by prolonged stimuli, the so-called morphological changes, also facilitate more efficient animal adaptation to the substrate, as occurs with cryptic fish.

Pigmentary system

The functions of chromatic patterns

Ectothermic animals can present chromatic adaptation to various situations such as light intensity, background color, hydrostatic pressure, temperature, osmosis, etc., (Bagnara and Hadley, 1973; Ligon and McCartney, 2016). The expression of different color patterns can also be associated with temporal and spatial alterations in animal behavior as well as mimetic and sexual changes (Dominey, 1981; Price et al., 2008). Furthermore, pigmentation can also express the conditions of the organism. Some pigments or their predecessors cannot be synthesized, and so must be obtained through the diet. In many cases, the total number of pigments expressed by the animal is related to success in foraging and the physiological

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efficiency of each individual, or in other words, the color level of an individual can indicate the state of its health. Moreover, besides this variation in expression based on diet, pigments can also be expressed in accordance with the ontogenetic state of the organism (Rodrigues et al., 2009). This flexibility in color-pattern expression shows that this functionally can go beyond simple static signaling, such as the identity of the species, its gender, or the stage of development, but can also underlie highly elaborate behavior.

The most common response of adaptation to the substrate observed in fish chromatophores, known as "protectionist color," is an important survival strategy in avoiding possible predators. For example, when exposed to a dark background, light-absorbing chromatophores disperse contained pigments (Figs. 7.1 and 7.2). The inconspicuous patterns so obtained are generally related to cryptic behavior, the variation in color and pattern representing a survival strategy.

In freshwater fish, descriptions of behavior related to color patterns have been almost exclusively restricted to cichlids. In most cases, apparently color change in these fish is related to specific behavior, such as camouflage, communication, etc. These patterns are molded by various forms of selective pressure, as imposed by predators, prey, competitors, and sexual partners. Some of these interactions favor reduction in visibility, such as cryptic coloring, whereas others induce the opposite. Plasticity in color patterns and the associated behavior appear to be a common solution for balancing opposite pressures. Cryptic patterns may be obtained by modification in color patterns, behavior, or both. For example, adjusting the moment of interruption of response to changes in the spectral quality of environmental light, and due to differences in the visual capacity of *Poecilia reticulata* and their predators, makes it possible for these fish to reduce visibility to predators and increase it for their associates (Grether et al., 2004).

Studies have shown that some elements of color are specifically in syntony with the visual capacity of cospecific individuals, but distant from those that are visible to predators. This is known as private signaling. Subaquatic environmental light is dynamic, both temporally as well as spatially, and the visual capacity of fish also varies dramatically. Some species are tetrachromatic and possess the capacity to see ultraviolet light or that from the red spectrum, whereas others are partially or completely blind in relation to what human beings are able to see.

Conspicuous color patterns may be classified as permanent, seasonal, or transitory. This tends to be permanent in species in which reproduction periods are prolonged, or that possess fixed territories all year round. Many cichlid species possess a permanent pattern that can be associated with competition and dispute for sexual partners (Seehausen et al., 1999). Seasonal patterns generally occur in species with a restricted reproduction period. For example, many salmonids change from a silver coloring to a bright red when dispersing carotenoids in erythrophores during the reproductive period (Korzan et al., 2008). In *Paralabrax clathratus*, only sexually mature individuals develop a bright color, whereas in *Cyprinodon pecosensis*, only males capable of establishing reproduction territories develop a nuptial color (Korzan et al., 2008).

Transitory patterns are a flexible mode of expression subject to neural and/or hormonal controls that are capable of change in a matter of seconds. Thus, due to their nature, with such fast activation or inactivation, they are particularly difficult to study. Melanophores are almost always involved in rapid color change in fish, also through neural and/or hormonal mechanisms. There are, however, examples of nonmelanic and fast color change. For example, the color of the tail fin in *Macrorhamphosus scolopax* changes to bright red during courtship through the dispersion of carotenoid-containing vesicles in the erythrophores (deOliveira et al., 1996).

Other color patterns may be related to specific behavior, such as the dorsoventrally aligned body pattern present in individuals' bodies. This pattern of color is usually associated with intraspecific behavior.

In a phylogenetic study of cichlids (Seehausen et al., 1999), the focus was on which behaviors could be associated with the resources and elements of a certain color pattern. The appearance of black stripes in these fish could be associated with the occupation of structured habitats instead of open water, where individuals are more susceptible to territoriality, and resources are aggregated in space and time, hence implying intense intraspecific competition for both resources and partners.

Stripes are cranial-caudally aligned patterns that generally occur in some social species such as zebras, neons, and some of the hemulids and serranids, among others. There are various hypotheses as to why fish that live in shoals present stripes. One is that they can distort the characteristic outline of the organism, thereby making it somewhat inconspicuous (Seehausen et al., 1999).

Other color-pattern modes have been clustered into a group known as "conspicuous color." This in turn is divided into two items: protective mimicry, as when a species adopts the characteristics of another that has either a disagreeable taste or is poisonous, and competitive mimicry. While protective mimicry is well understood in various groups of vertebrates and invertebrates, this latter classification of mimetic interaction has been proposed by Rainey and Grether (2007).

Protective mimicry has been reported in various fish species. All toxic fish of aposematic color are models for mimicry by nontoxic species. There are many cases, especially in tropical reefs, where the mimicker presents the general body shape and bright color of its model. Eventually, aposematic species may live and forage cryptically, although presenting an

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outstanding display when in danger. Some families of Mediterranean fish, such as the Trachinidae and Uranoscopidae, possess venomous spines and an eye-catching spot on the first dorsal fin. While foraging discretely buried in the substrate, individuals can show this distinctive mark when threatened. On the other hand, edible members of the family Solidae also possess a modified pectoral fin with a very similar black spot, possibly to avoid attacks (Bedini et al., 2003).

As a case of competitive mimicry, juvenile surgeon fish (*Acanthurus pyroferus*) can be cited as an example through their similarity to the angel fish (*Centropyge vrolikii*). This presumably facilitates their foraging in territories dominated by *Plectoroglyphododon lacrymatus* because these tolerate angel fish, but repel adult surgeons (Munday et al., 2003). Intraspecific competitive mimicry generally comprises an alternative male reproductive strategy. In many of the species that employ such, individual males try to resemble conspecific juveniles and females as a form of obtaining access to the female's territory. Association of melanic color with various types of behavior has been observed not only in fish, but also among other vertebrates. It is likely that this involves pleiotropy, the physiological basis of behavior, and melanic color, all of which are linked through the neuroendocrine system.

Pigment cells and pigments

There are basically five types of chromatophores in certain invertebrates (crustaceans and cephalopods) and ectothermic vertebrates (fish, amphibians, and reptiles). The chemical nature of the pigments and the color that these confer to pigment cells were used for classifying these organisms (Fujii, 2000).

Melanophores, the most amply distributed chromatophores, are responsible for a large part of vertebrate pigmentation. These cells synthesize two types of melanin, namely eumelanin (brown and black) and pheomelanin (yellow or red). Both are dendritic in appearance. Ectothermic vertebrates do not produce pheomelanin (Fujii, 2000). Both are polymers derived from the aminoacid tyrosine in a process denominated as melanogenesis. Synthesis begins with enzymatic oxidation from L-tyrosine to L-Dopa and oxidation from L-Dopa to dopaquinone, through the action of tyrosinase, the key enzyme in the process. The spontaneous transformation of dopaquinone into leucodopachrome and dopachrome is the beginning of a biochemical cascade leading to the formation of a brownish-black pigment called eumelanin. The conjugation of dopaquinone with cysteine or glutathione gives rise to cysteinyldopa and glutathiondopa, both of which pass through a series of transformations, finally leading to a reddish-yellow pigment called pheomelanin (Fig. 7.3) (Bagnara and Hadley, 1973; Ligon and McCartney, 2016).

Melanophores represent the most common type of chromatophore in fish, amphibians, and reptiles. The chromatosomes present in these cells (melanosomes) are organelles that contain pigment granules. They are either circular or slightly elliptic with a diameter of around 0.5 μm. When compared with other types of chromatophores, melanophores generally present higher and more active mobility. Rapid melanophore aggregation or dispersion occurs as a response to neural or

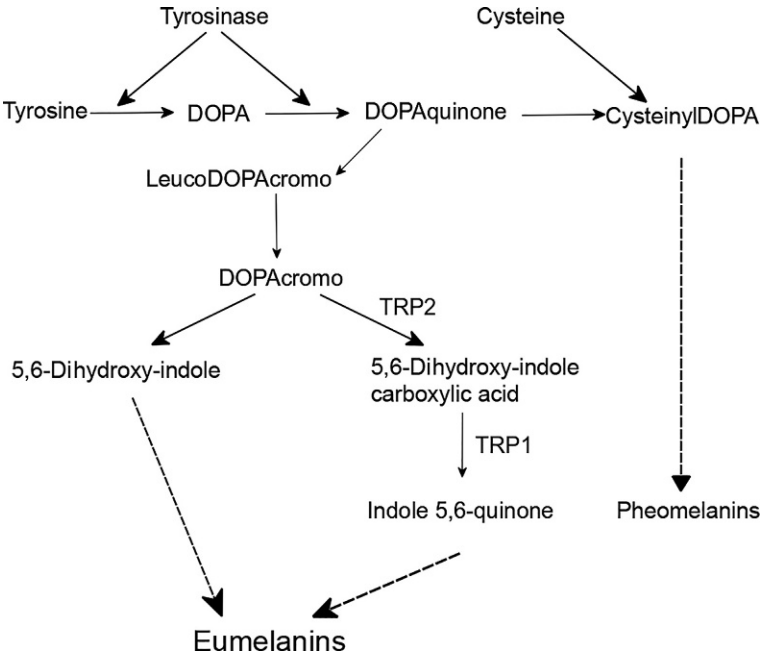

FIG. 7.3 Melanin synthesis pathway (original by Luciana Andrade).

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hormonal stimulation. Hence, melanophores play an important role in the quick color change shown by most fish. The in vivo and in vitro manipulations of these cells are relatively easy, and are the reason why they have been much used in studies of those factors that control color and chromatophores as a whole (Bagnara and Hadley, 1973; Fujii, 1993; Fujii and Oshima, 1994; Ligon and McCartney, 2016).

The dendritic-shaped and reddish-colored erythrophores contain pigments of the carotenoid and pteridine type. The most common chromatophores are found in reddish-colored fish, and they are also widely spread among those freshwater and coral fish that present red skin spots. They generally present the same morphological characteristics as the melanophores. The color of the carotenoids and pteridines, unsaturated compounds, varies from yellow to red. In several cases, it has been noted that erythrophores actively participate in skin-color change. Carotenoid vesicles and pterinosomes move toward the cell body or the cell perimeter in response to certain signals. Erythrophores are responsible for the eye-catching and lively color found in several groups of fish. Animals, by not having the capacity to synthesize carotenoids, obligatorily obtain them from food. Even so, they are capable of producing pteridines and purines (Bagnara and Hadley, 1973; Fujii, 1993; Fujii and Oshima, 1994; Ligon and McCartney, 2016).

The yellowish-colored xanthophores are also dendritic chromatophores. They are generally smaller than melanophores and erythrophores. Even though many authors do not distinguish them from erythrophores, certain morphological characteristics as well as their localization may be used in their differentiation. More often associated with melanophores, their function is to give a yellowish tone to black, thereby generating brown, which is fairly common in fish tegument. As in the erythrophores, the color-induction compounds are carotenoids and pteridines, although in different proportions, with predominance of the water-solvent pteridines (Bagnara and Hadley, 1973; Fujii, 1993; Fujii and Oshima, 1994).

Even though leucophores, as with the previously mentioned chromatophores, are also dendritic in appearance, their optic properties are entirely different. Instead of absorbing, they reflect light by way of cytoplasmic purine granules. The extremely restricted distribution of these cells is almost exclusively limited to teleosts. The reflection-inductive, globe-shaped, membrane-covered organelles, or leucosomes, reflect incidental light in all directions. Even though less numerous than in other chromatophores, they are generally of the same size or even larger. Structural characteristics are fundamentally the same. Leucophores are often to be found overlaying a melanophore (Bagnara and Hadley, 1973; Fujii, 1993; Fujii and Oshima, 1994; Ligon and McCartney, 2016).

The metal-colored iridophores possess purines (guanine) arranged in the form of crystalline plates by which they are capable of reflecting incident light. The distance between the plates is even throughout, and then changes in reflection occur through the simultaneous alteration of this distance. They are very common in the skin of fish, especially in the abdominal region, which generally presents a silver or whitish color. They can also occur when associated with melanophores (Bagnara and Hadley, 1973; Fujii, 1993; Fujii and Oshima, 1994; Ligon and McCartney, 2016).

Purines are practically water-insoluble. They are efficiently active in reflecting light and, according to the pigment structural arrangement, confer an iridescent blue, silver, golden, or white color to fish. These colors are derived from at least one of two phenomena: light interference or diffraction (Bagnara and Hadley, 1973; Fujii, 1993; Fujii and Oshima, 1994; Ligon and McCartney, 2016).

During interference, incident light reflects at different wavelengths due to the arrangement of reflective structures, which in iridophores comprise guanine crystal plates. Depending on the distance between these plates, they reflect light in such a way that part of the spectrum is either reduced or simply canceled out. Thus, what is actually seen is the sum of the remaining wavelengths, and numerous teleosts present iridescent body areas (Bagnara and Hadley, 1973; Fujii, 1993; Fujii and Oshima, 1994; Ligon and McCartney, 2016).

During diffraction, there is scattering of the shortest wavelengths of incident white light by very small particles. Many teleosts, such as discus, neons, and others, present brilliant blue-colored patches on the body. Within these areas, incident light is scattered by guanine crystalline plates. The intensity and shade of the blue depends both on the angle of these plates in relation to incident light and the distribution of iridophores in the skin. Some authors consider that in teleosts, shades are not derived from scattering but from interference in multiple layers (Bagnara and Hadley, 1973; Fujii, 1993; Fujii and Oshima, 1994; Ligon and McCartney, 2016).

Mechanisms for moving pigment granules-motor proteins

Aspengren et al. (2009) revised the knowledge on pigment-granule shifting in vertebrates. In studies of melanophores in fish, it was shown that melanosome shifting in these cells is dependent on microtubules (MTs). Exposure to low temperatures, high pressure, or certain types of drugs can jeopardize their mediation in granule aggregation and dispersion. Furthermore, MTs are also crucial for maintaining the spherical shape of pigments in aggregated melanophores. Apart from

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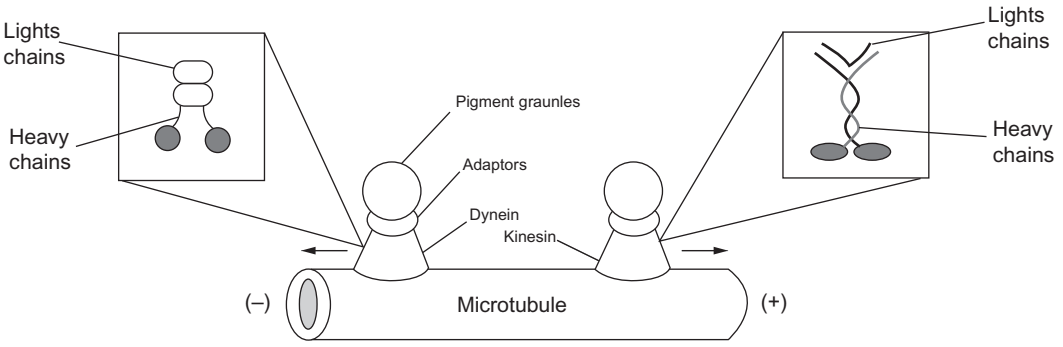

FIG. 7.4 Diagram of the granule movement mechanism. See text for process description (original by Luciana Andrade).

microtubules, movement in chromatophores is also dependent on the intracellular presence of shift proteins, especially dynein and kinesin (Fig. 7.4).

Cytoplasmic dynein is typically involved in movement toward the center of the cell; in other words, in the aggregation of pigment granules. A homodimer is generally formed by two identical heavy chains that comprise the two motor domains as well as various other small and medium chains. The heavy chains form the heads, which are the ATP catalytic nuclei attachable to microtubules. The other chains form the tails, and are attachable to structures that will be carried (Fig. 7.4). Some dynein blockers also inhibit melanosome aggregation in fish, whereby indirect proof that dyneins play an important role in melanosome aggregation. When injecting specific antibodies against the intermediary dynein chain in cod melanophores, there was complete blockage of aggregation without any effect on dispersion. They also play an active role, both in maintaining the spherical shape of the central mass in pigment cells and in the homogeneous distribution of dispersed melanosomes.

There are at least nine different types of kinesins. Most are motor proteins that move toward the sites of MT polymerization (the "plus end"). They are also involved in organelle shifting from the center of the cell to the periphery. Basically, kinesins are tetramers comprising two heavy chains containing the motor domains, associated with two light chains that adjust to the structures that will be carried by microtubules (Fig. 7.4). To date, it has not been determined with certainty which kinesins are involved in pigment granule dispersion in fish.

Besides MTs, studies of teleosts have shown the participation of actin filaments in pigment-granule shifting. Even with MT depolymerization, pigment shifting is not completely blocked. When treated with substances that derange actin filaments, teleost chromatophores present an increase in the degree of aggregation or dispersion. Recent studies have indicated that this actin-dependent movement, important for the homogeneous distribution of granules in cell processes, involves the participation of a motor protein from the myosin family, probably myosin-V, whose activity is dependent on ATP.

Chromatic adaptation

Even though a chromatic pattern is determined genetically, animals are capable of changing color in response to environmental stimuli, thereby facilitating adaptation to various situations. These changes are classified as either morphological or physiological.

Morphological change occurs in all animals, including birds and mammals. It is derived from alterations in the amount of pigments in pigment cells or the number of pigment cells (chromatophores) themselves. The changes, although extremely slow and taking from days to weeks until completion, are long-lasting. This is what occurs, for example, during sun exposure. Ultraviolet radiation stimulates melanin synthesis in melanocytes, with the subsequent transfer of pigment granules to keratinocytes in a process commonly known as sun burning. This plays a very important role in skin-cell DNA protection against damage caused by this form of radiation. A further example is the case of juveniles of *Sphyrna lewini* (hammerhead shark), which primarily, as a means of protection against predators and to facilitate obtaining food, stay in shallow-water reef environments, thereby leading to higher sun exposure. Consequently, they present a darker skin when compared to adults, which live in deeper waters (Lowe and Goodman-Lowe, 1996).

The other type of color change, the physiological, occurs in certain invertebrates such as cephalopods and crustaceans as well as ectothermic vertebrates. In the latter, this form of change is based on granule-shifting inside chromatophores. This extremely fast process is completed in a matter of seconds or, at the most, minutes. The animal becomes bright-colored when the granules in dark-colored chromatophores (melanophores and erythrophores) aggregate on the cell body, leaving them as cell patches devoid of pigments. Simultaneously, the granules of bright-colored chromatophores (xanthophores and

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leucophores) are dispersed through projections. When the animal becomes darker-colored, pigments in the former are dispersed, whereas in the latter they are aggregated (Figs. 7.1 and 7.2). The mechanisms involved in physiological color change are extremely complex and have disappeared in birds and mammals, which present only the morphological form (Bagnara and Hadley, 1973; Ligon and McCartney, 2016).

Regulation of chromatic adaptation

Primary response

There are two forms of response in physiological color change, primary and secondary. In a primary response, chromatophores respond directly to light, independent of nervous and endocrinal system involvement. In most of the invertebrates, including sea urchins and crustaceans, light induces pigment dispersion, whereas in amphibians and fish there can be either aggregation or dispersion. In cultures, when melanophores of *Danio rerio* are exposed to direct light, melanosomes aggregate. The higher the light intensity, the higher the aggregation/dispersion. This also occurs with xanthophores of the same species when so exposed, with aggregation of carotenoid granules. Likewise, in other teleost species such as *Xiphophrorus maculatus*, *Oryzias latipes*, *Rhodeus ocellatus*, *Zacco temmincki,* and *Oreochromis niloticus*, pigment response to direct light is the same to the same degree. This presupposes the presence of one or more as yet unidentified opsins in these pigment cells (Oshima, 2001).

Secondary response

As mentioned above, various hormones and neurotransmitters are involved in the control of pigment cell migration in ectothermic vertebrates, of which α-MSH and MCH (the latter only in teleosts and holosteis), catecholamines (both of humoral and nervous origin), and melatonin are undoubtedly the most important and well known as regards mediation in hormone pigment-cell regulation. The participation of others, such as endothelins, was investigated in a limited number of species. On linking with their respective transmembranous receptors, a cascade of intracellular events is unleashed, which results in pigment-granule shifting (Fujii and Oshima, 1994; deOliveira et al., 1996; Nery and Castrucci, 1997; Fujii, 2000).

Melanocyte-stimulating hormone α-MSH

α-MSH, a peptide comprised of 13 amino acids, originates from the cleavage of a precursor of high molecular weight, namely proopiomelanocortin (POMC), which also gives origin to betalipotropin, ACTH, and other peptides (Mountjoy et al., 2003; Jégou et al., 2006). With some exceptions, such as humans and birds, α-MSH is produced and liberated by the *pars intermedia* of the hypophysis.

Apart from hypophysis cells, POMC expression occurs in hypothalamic neurons, keratinocytes, and Langerhans cells as well as other tissues (Takahashi and Kawauchi, 2006; Mountjoy et al., 2003; Jégou et al., 2006). From the physiological point of view, in all the vertebrates studied, α-MSH is the most relevant pigment hormone through stimulating pigment granule transport, melanin synthesis, and cell proliferation (Sherbrooke et al., 1988; Takahashi and Kawauchi, 2006). In teleosts, this peptide induces pigment dispersion; in other words, the darkening observed in all the species studied (Fujii, 2000; Takahashi and Kawauchi, 2006). Although at least five subtypes of receptors have been cloned and sequenced, the expression of only one, namely melanocortin-1 (MC1-R), is encountered in pigment cells (Mountjoy et al., 2003; Jégou et al., 2006). The others are more amply distributed, having been identified in the cells of various tissues, including the central nervous system but not in the skin (Mountjoy et al., 2003; Jégou et al., 2006).

The MC1-R and other receptor subtypes have been identified in teleosts, with interspecific differences. For example, *D. rerio* has six genes for melanocortin receptors with two copies of mc5r, whereas *Takifugu rubripes* and *Tetraodon nigroviridis* have four (Logan et al., 2003b; Schiöth et al., 2005). In other teleosts, several genes for α-MSH have also been cloned (Metz et al., 2006; Schiöth et al., 2005). The MC3-R receptor, which in mammals is the receptor whose preferential link is α-MSH, has not been found among the teleosts (Takahashi and Kawauchi, 2006).

The melanocortins modulate teleost pigmentation, probably by linking to the MC1-R receptor (Logan et al., 2006). The receptor gene has been identified in *T. rubripes* and *T. nigroviridis* as well as in *D. rerio* and *Oreochromis mossambicus* (Metz et al., 2006; Logan et al., 2003b; Schiöth et al., 2005).

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Melanin concentrating hormone (MCH)

MCH is a highly lipophilic cyclic heptadecapeptide that possesses a disulfate bridge between positions 5 and 14. Enami (1955) was the first to propose the existence of a lightening hormone. However, since the 1930s, various researchers have been working on identifying an active antagonic principle for α-MSH. The sequence of amino acids of this hormone was finally obtained by Kawauchi et al. (1983) in the teleost *Onchorhynchus keta*. This peptide is linked to skin pigment cells, thereby inducing the aggregation of pigment granules or their lightening. However, this function is noted only in teleosts and holosteis.

Two subtypes of MCH receptors have been found: MCH-R1, which has been reported in all the species studied, and MCH-R2, apparently present only in some mammals, and belonging to the large family of receptors linked to the G protein (GPCRs), characterized by presenting seven well-conserved α-helices that cross the plasma membrane and are directed in such a way that the aminoterminal region is extracellular and the carboxiterminal intracellular. However, on the contrary to MCH-R1 that is extremely well conserved in several species, MCH-R2 appears to have a definite function only in humans because, even though other species do possess this receptor and with mutually highly homologous sequences, functional expression has not been conserved during evolution (Lembo et al., 1999; Saito and Maruyama, 2006; Nahon, 2006; Chung et al., 2011).

In fish, three MCHR sequences have been identified, one in *D. rerio* and two in *Fugu rubripes*. Phylogenetic analysis of these fragments infers that initial duplication of genes codified for MCHR must have occurred early in evolution. These two species possess orthologue sequences for *mchr-1* and *mchr-2* genes (Logan et al., 2003a, b). The authors also presuppose that the third gene for the *D. rerio* receptor probably arose from a late duplication event during teleost evolution. MCH receptors have been characterized in two other species: *Verasper moseri* with R1-356-aa and R2-345aa (Takahashi et al., 2007) and *Carassius auratus* with R1-359aa and R2-342aa (Mizusawa et al., 2009). MCH-R2 receptors mediate the effects of MCH in color change in response to the background color in teleost *V. moseri*. (Takahashi et al., 2007).

Apparently, this neuropeptide has been well conserved throughout evolution. Notwithstanding, its role seems to diversify among several species: neurotransmitter and neuromodulator in the vertebrate brain; circulating hormone in fish; and probable paracrine/endocrine agent in certain peripherical tissues (Kawauchi et al., 1983; Sherbrooke et al., 1988; Isoldi et al., 2004; Kawauchi and Baker, 2004; Nahon, 2006). In the teleosts, it inhibits α-MSH and ACTH secretion by hypophysis and regulates food ingestion (Takahashi et al., 2004; Shimakura et al., 2008; Volkoff et al., 2005). There is evidence that the pigment function may have emerged in a more recent stage in teleost and holosteis evolution because its lightening activity has only been observed among these animals (Sherbrooke et al., 1988; Kawauchi and Baker, 2004), whereas evidence of this function in other groups of vertebrates is virtually nonexistent (Kawauchi and Baker, 2004; Cardinaud et al., 2004).

All the studies to date show the MCH efficiency in pigment-aggregation induction not only in melanophores, but also in erythrophores, xanthophores, and leucophores (Kawauchi and Baker, 2004; Fujii, 2000).

Catecholamines

Catecholamines, produced and liberated by both the sympathetic nervous system and the adrenal medulla, are powerful agonists in ectothermic vertebrate pigment cells. Depending on the type of receptor activated, α or β, the response can be either lightening of the animal by the aggregation of melanophore, erythrophore, or xanthophore pigment granules, or darkening by their dispersion (Aspengren et al., 2009; Fujii and Oshima, 1994; Fujii, 2000, deOliveira et al., 1996).

The catecholamines comprise a catechol (a benzenic ring with two hydroxyl groups) and an amine lateral chain. The most important catecholamines in pigmentation physiology are epinephrine, produced and liberated by the interrenal gland, and norepinephrine, produced and released by the postganglionic neurons of the sympathetic division of the autonomous nervous system (Rang et al., 2007). The catecholamines are synthesized from the aminoacid L-tyrosine, retained by adrenergic neurons, and initially converted into L-DOPA by the enzyme thyroxin hydroxylase. As observed in the synthesis of melanin, this step is the main control point in the process. However, thyroxin hydroxylase should not be confused with thyronase, which, besides being a hydroxylase, is also an oxidase (Prota, 1992).

Studies with agonists and selective antagonists have demonstrated that there are two main subtypes of adrenoceptor α, α_1, and α_2, and three subtypes of adrenoceptor β, β_1, β_2, and β_3, all of which are attached to a G protein (Rang et al., 2007). Certain studies, especially of teleost chromatophores, have identified the subtypes of α adrenoceptors that mediate aggregation through catecholamine action (Fujii and Oshima, 1994). Notably agonists as well as selective antagonists for adrenoceptors α_2 are more effective than those for α_1 (Andersson et al., 1984; Morishita, 1987; Morishita et al., 1993). Karlsson et al. (1987) analyzed the aggregative response in seven teleost species belonging to five different families. Notably,

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yohimbine (selective antagonist α_2) was generally more effective in reducing the response to norepinephrine in comparison to prazosin (selective antagonist α_1) These results lead the authors to classify them as α_2. In 1993, Morishita et al. (1993), using selective antagonists, characterized the adrenoceptors present in *C. auratus* melanophores as α_2. Svensson et al. (1993) cloned this adrenoceptor, and subsequent analysis showed there to be greater similarity between this and that of mammals than between teleosts and the nearest groups.

Yamada (1980) was the first to characterize β adrenoceptors in teleosts by identifying the β_1 subtype in the leucophores of *O. latipes*. Subsequently, Morishita et al. (1985) showed that in melanophores of the same species, there are only β_2 subtypes. Finally, in 1990 Katayama et al., when using agonists and selective antagonists, showed the presence of both subtypes in melanophores of *Tridentiger obscurus*, with β_2 predominant.

Melatonin

Melatonin was identified by Lerner et al. (1960) as the substance that produces changes in the color of the skin in amphibians, and is released by the pineal gland during darkness. In the vertebrates, it is also synthesized by the retina, where it plays a paracrine role by participating in adaptative processes in nocturnal vision (Tosini and Fukuhara, 2003).

This indolamine is synthesized from the amino acid tryptophan, which is obtained from the bloodstream and transformed into 5-hydroxytryptophan (5-HTP) through the action of the enzyme tryptophan hydroxylase 1 (TPH1), which is more active in darkness. 5-HTP is then decarboxylated by the enzyme 5-HTP decarboxylase, thereby forming a serotonin. This, in turn, is metabolized to *N*-acetylserotonin (NAS) by the action of the enzyme arylalkylamine *N*-acetyltransferase (AA-NAT). Subsequently, NAS is used as a substrate by the enzyme hydroxy-indol-*O*-methyltransferase (HIOMT), with melatonin as the final product (Simonneaux and Ribelayga, 2003).

The limiting step in melatonin synthesis is the conversion of serotonin to *N*-acetylserotonin. This conversion occurs only in darkness, and is interrupted at the end of the night or when there is a flash of light in the dark. During darkness, the enzyme *N*-acetyltransferase is a hundred times more active in mammals. The signal that activates this mechanism is a rise in the release of noradrenalin from a sympathetic route controlled by suprachiasmatic nuclei that are the seat of the biological clock (Simonneaux and Ribelayga, 2003). Otherwise, in teleosts the pineal also directly responds to incident light (Fujii and Oshima, 1994; Fujii, 2000).

According to differences in pharmacological profile and subtype cloning, the linkage sites for melatonin are classified between MT_1, MT_2, and Mel_{1c} ((high affinity receptors) and MT_3 (low affinity receptor) (Dubocovich et al., 1998; Shiu and Pang, 1998). The Mel_{1c} receptor has already been cloned in *D. rerio* (Reppert et al., 1995).

In teleosts and amphibians, melatonin plays a role in controlling skin color through chromatophores (Fujii and Oshima, 1994). In various teleost species, there is a circadian rhythm in color change, with nighttime lightening (Fujii and Oshima, 1994).

Fujii (1961) was the first to demonstrate melatonin aggregation activity in the melanophores of the gobioid fish *Chasmichthys gulosus*. Afterward, several authors showed that this indolamine aggregates pigment granules in melanophores, xanthophores, erythrophores, and leucophores, besides altering the slope and spacing between the guanin crystalline plates in iridophores, thereby modifying the tones of metallic colors.

Differences also exist between species as to sensibility to melatonin (Fujii et al., 1993; Fujii and Oshima, 1994; Filadelfi et al., 1996). Matsumoto et al. (1978), for example, described pigment aggregation in primary cultures of erythrophores of *Xiphophorus helleri*. Iridophores of *Paracheirodon innesi* and *Chrysiptera cyanea* were found to respond by intensification of metallic colors (Fujii and Oshima, 1994), and there was aggregation of leucosomes in leucophores of *O. latipes* (Obika, 1988).

On the other side, there are species in which there is no pigment-cell response to melatonin, such as *Fundulus heteroclitus*, *P. reticulata,* and *C. carassius*. There are still others, such a *Nannostomus beckfordi*, in which melanophores in specific regions of the body aggregate melanosomes, whereas in others there is no response, and further still, in the same animal, other melanophores respond with pigment dispersion at night (Fujii and Oshima, 1994).

Endothelins

Besides the above-mentioned hormones and neurotransmitters that are undoubtedly animal pigmentation modulators, still other hormones, not originally known by their role in pigmentation, have been identified as capable of pigment cell regulation in vertebrates. Among these, the endothelins are outstanding.

Endothelins are cyclic peptides from 21 amino acids that, in mammals, possess three isoforms: ET-1, ET-2, and ET-3 (Kochva et al., 1993). They originate from a pre-pro-hormone denominated prepro-ET (Inoue et al., 1989). ET-1, a strong

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vasoconstrictor, seems to be exclusively synthesized in endothelial cells, whereas ET-2 and ET-3 are produced in various tissues (Davenport and Maguire, 2006).

These hormones have several functions, especially in vasoconstriction. Furthermore, they induce increased proliferation in several types of cells (Kedzierski and Yanagisawa, 2001), and in human melanocytes, cell proliferation (Yada et al., 1991), and melanogenesis (Yada et al., 1991; Tada et al., 1998).

Besides being active in vasoconstriction in ectothermic vertebrates (Poder et al., 1991), ET-1 participates in controlling blood circulation in teleosts and elasmobranch fish (Zaccone et al., 1996). Nevertheless, even though in mammals the peptide molecules of the endothelins are relatively well characterized, in the other vertebrates little is known about biological effects. Hormone activities are mediated by three types of receptors, each of which, in turn, possesses a different specificity for one of the three peptides (Karne et al., 1993). The two receptor subtypes, ET_A and ET_B, identified in mammals as members of the superfamily of receptors linked to the G protein (GPCRS), are widely distributed in several tissues (Arai et al., 1990; Sakurai et al., 1990). A third, ET_C, has been cloned and characterized from dermic melanophores of the amphibian *Xenopus laevis* (Karne et al., 1993).

Endothelins are capable of inducing pigment migration in teleosts and amphibians (Fujii et al., 1993; Karne et al., 1993; Camargo et al., 1998). A characteristic of teleost skin is numerous sites with ET secreting cells active in the final adjustment of hues (Fujii, 2000), and inducing aggregation (lightening) or dispersion (darkening), depending on the intracellular signaling pathway that is being modulated (Nery and Castrucci, 1997; Isoldi et al., 2004). In some species of teleosts, ETs also induce pigment aggregation in melanophores (Fujii et al., 1993; Filadelfi et al., 1996; Ramanzini et al., 2006) and xanthophores (Murata and Fujii, 2000), and dispersion in leucophores (Fujita and Fujii, 1997).

Action mechanisms (intracellular signaling pathways)

Structurally, the already-described hormones and neurotransmitters are members of the largest superfamily of receptors known, those linked to the G-GPCRS protein. These receptors are composed of seven transmembranous domains and intra- and extracellular tabs. The G protein is a heterotrimeric enzyme with three subunits, α, β, and γ. The linkage of an agonist to these receptors leads to the exchange of a GDP molecule for a GTP, and the dissociation of the α subunit from βγ. The α subunit possesses GTPase activity and is then free to activate membrane enzymes or ionic channels, although in some cases βγ plays the active role. The effector activity ends when hydrolysis of the GTP linked to the α subunit occurs, thereby leading to its recombination with βγ (Fig. 7.5). There are various types of G-proteins that interact with several receptors and control numerous intracellular signaling pathways (Rang et al., 2007).

GPCRSs are classified by α subunit signaling characteristics, and can be grouped into three classes, G_s, G_i/G_o, and G_q, the main ones of which are shown in Fig. 7.6 (Rang et al., 2007). G_s stimulates adenylyl cyclase, thereby leading to an increase in intracellular levels of cyclic 3′,5′-adenosin-monophosphate (AMP). G_i/G_o inhibit adenylyl cyclase, activate phosphodiesterases, and regulate K^+ and Ca^{2+} channels. G_q activates phospholipase C (PLC), thereby leading to an increase

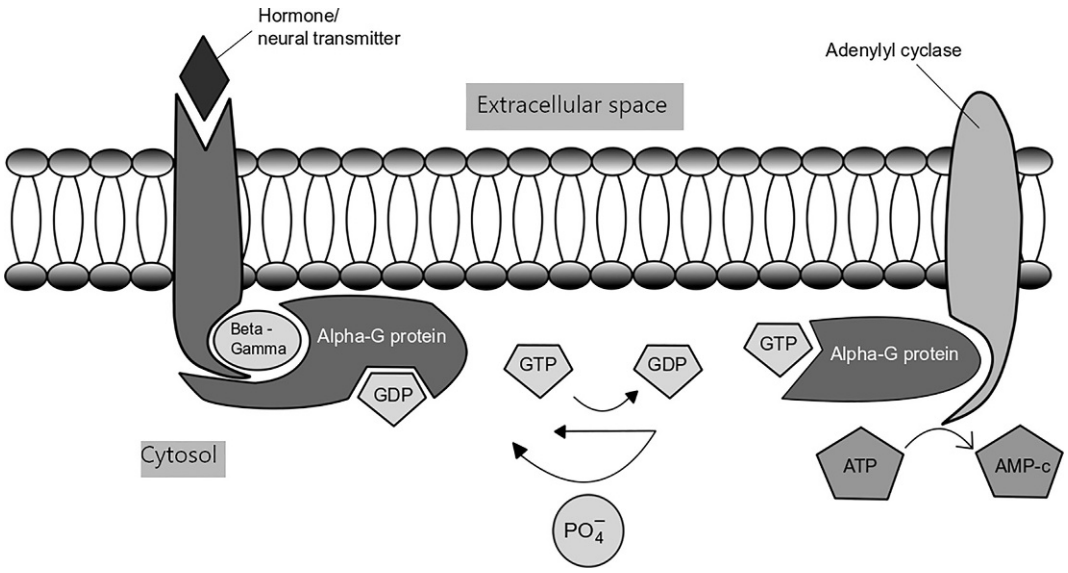

FIG. 7.5 Diagram showing the receptors coupled to protein G. See text for process description (original by Luciana Andrade).

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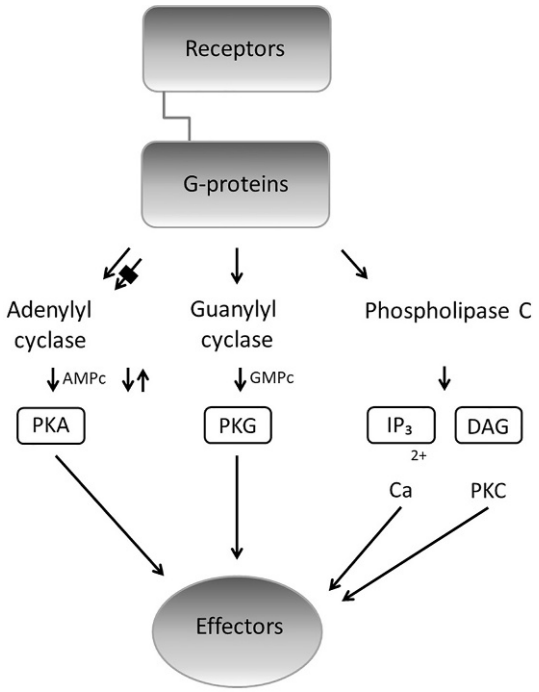

FIG. 7.6 Diagram showing some of the signaling pathways triggered by the activation of G proteins (original by Vanessa Seiko).

in intracellular inositol triphosphate (IP_3) and diacylglycerol, from whence the release of Ca^{2+} from intracellular reservoirs (mainly the endoplasmatic reticulum), besides activation of protein kinase C (PKC). Ca^{2+} can also join with calmodulin, and this compound eventually activates several proteins, including a Ca^{2+}/calmodulin-dependent PKC isoform. At the present, many details of these pathways (and even others not mentioned above) are known, but in view of the complexity of the theme, in this chapter we have kept to what is actually known about heterothermic-vertebrate pigment cells.

Pigment dispersion is induced by α-MSH or catecholamines when these are linked to β adrenoceptors. In both cases, their receptors are linked to a Gs protein that activates the enzyme adenylyl cyclase, thereby leading to an increase of intracellular AMPc levels and PKA activation (deOliveira et al., 1996; Nery and Castrucci, 1997). Probably, this is the α-MSH activated mechanism in the baiacus *T. rubripes* and *T. nigroviridis, D. rerio*, the tilapia *O. mossambicus*, and the swamp eel *Synbranchus marmoratus* (Castrucci et al., 1997; Metz et al., 2006; Logan et al., 2003a; Schiöth et al., 2005). Likewise, the linkage of catecholamines to β adrenoceptors activates this pathway in the leucophores of *O. latipes* and in the melanophores of *T. obscurus* (Yamada, 1980; Morishita et al., 1985; Katayama et al., 1990).

Certain wavelengths also induce pigment dispersion in teleosts (Oshima, 2001), although the active mechanism is as yet little understood. Studies with melanophores of *X. maculatus*, xanthophores of *O. latipes*, and erythrophores of *O. niloticus* presuppose that dispersion would be mediated by the G_s protein through an intracellular increase of AMPc (Kasukawa and Oshima, 1987; Wakamatsu, 1978; Oshima, 2001). In tadpoles of the amphibian *X. laevis*, there is an increase in intracellular AMPc levels in isolated melanophores to which visible light had been applied (Daniolos et al., 1990) In these animals, dispersion is mediated through a protein linked to the membrane similar to rhodopsin, denominated melanopsin, which activates G_s (Provencio et al., 1998). Contrary to that, in an immortalized line of melanophores of this same amphibian, it was shown that light-induced dispersion is probably mediated by the G_q protein through an increase in intracellular IP_3 and Ca^{2+} levels (Isoldi et al., 2004).

Pigment aggregation is induced by catecholamines, MCH, melatonin, and endothelins. Signaling pathways involve G_i/G_o proteins, with a decrease of intracellular AMPc levels and/or G_q, which, together with the participation of IP_3, diacylglycerol, and Ca^{2+}, activate PKC. Apart from these compounds, certain wavelengths, distinct from those that induce dispersion, can also induce aggregation (Oshima, 2001).

Catecholamines induce aggregation through two adrenoceptor subtypes, α_1 and α_2. The signalizing pathways activated by either one or the other are different. α_2 activation inhibits adenylyl cyclase, diminishes AMPc levels, and inactivates PKA (Svensson et al., 1997; Nery and Castrucci, 1997; Aspengren et al., 2009). This signaling pathway was observed in melanophores of *Labrus ossifagus*, *O. latipes,* and *C. auratus* (Nery and Castrucci, 1997; Fujii, 2000). On the other hand, in some teleost species with participation of the α_1 adrenoceptor linked to G_q proteins, there is PKC activation and an

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increase in intracellular Ca^{2+} levels. Examples of this are *O. niloticus*, *O. latipes,* and *X. maculatus* (Nery and Castrucci, 1997). The participation of Ca^{2+}-calmodulin has been observed in the chromatophores of *O. mossambicus* (Thaler and Haimo, 1990). Nevertheless, one may also consider the possibility of the expression of both adrenoceptors occurring in the same pigment cell, from whence aggregation would be the result of AMPc reduction (G_i), PKC activation, and an increase in intracellular Ca^{2+} (deOliveira et al., 1996; Nery and Castrucci, 1997; Fujii, 2000).

In MCH, there is experimental evidence of two signaling pathways in teleost chromatophores. In *C. cyanea* (Oshima et al., 1986), *L. ossifagus* (Svensson et al., 1991), *Anguilla anguilla* (Cardinaud et al., 2004), and *D. rerio* (Logan et al., 2006), there is AMPc reduction, thus the receptor is linked to a G_1. protein. In *S. marmoratus* (Abrão et al., 1991) and in tumoral cells derived from erythrophores of *C. auratus* (Isoldi et al., 2004), the receptor, by linking to a G_q protein, activates phospholipase C, thereby generating IP_3 and diacylglycerol, elevating Ca^{2+} intercellular levels, and activating the Ca^{2+}/calmodulin-dependent PKC. In teleosts, two receptors were identified for MCH (Logan et al., 2003a; Takahashi et al., 2007), from whence came the possibility that these receptors activate different G proteins, although maybe both pathways are activated in the same pigment cell as suggested by Nery and Castrucci (1997). However, this has not yet been investigated.

Regarding ectothermic vertebrates, melatonin-activated signaling pathways have been investigated in more detail in the amphibians. In *X. laevis*, on linking to a high-affinity receptor, each melatonin activates G_i/G_o, thereby inhibiting adenylyl cyclase with AMPc reduction and PKA inhibition (Ebisawa et al., 1994). There is still another pathway that can be activated in this amphibian by the same receptor: $G_{\beta\gamma}$ activates phosphatidylinositol 3′-kynase (PI3-K), which in turn activates a phosphodiesterase, resulting in diminished AMPc levels (Andersson et al., 2003). When studying teleosts, Aspengren et al. (2003) investigated melatonin signaling pathways in melanophores of the cod *Gadus morhua*. Aggregation in this species is similar to that which occurs in *Xenopus* through a G_i/G_o pathway, but there is additionally an increase in intracellular Ca^{2+} also contributing to aggregation.

In the teleosts *N. beckfordi* and *N. trifasciatus*, melatonin induces pigment dispersion (Fujii, 2000). Furthermore, Nery and Castrucci (1997) proposed the presence of adenylyl cyclase, from whence receptor activation would contribute toward increasing AMPc. Nevertheless, there is no experimental proof of this transduction pathway.

There are several endothelin-activated signaling pathways that have generated much controversy in the literature. There are reports of G_i and G_s protein activation, IP_3 or calcium increase, no effects from calcium cytosolic concentration, and PKA, PKC, PLC, and MAPKs activation (Imokawa et al., 1997, 2000; Yada et al., 1991; Kobayashi et al., 2002).

In *D. rerio* melanophores, ET effects are inhibited by forskolin, implying that AMPc reduction induces hormonal efficacy (Kawabata and Oshima, 2001) in the same way as with other melanosome-aggregating or skin-lightening agents (Nery and Castrucci, 1997). On the other side, in normal human melanocytes, ET-1 induced melanogenesis increases AMPc and Ca^{2+} levels, reversible by pertussis toxin mediation (Imokawa et al., 1997). The signaling pathway of ETS-induced pigment dispersion in leucophores of *O. latipes* is unknown (Fujii, 2000).

When investigating ET-induced signaling in erythrophoroma cells of *C. auratus*, Filadelfi et al. (2009) demonstrated that some components of the classic pathways (PKC and AMP) in ectothermic vertebrates are important, though not exclusive, for hormonal mediation in this cell line, whereby the possible involvement of other pathways.

Final considerations

The exuberant colors of organisms have stimulated the aesthetic sense of humanity throughout the ages. This system, encountered in all classes of vertebrates and related to various functions, is fundamental for survival and adaptation. The scientific study of animal color really began around 150 years ago, with the investigation of color change in chameleons. At this time, several relevant questions on the physiology of chromatic adaptation had already been formulated. The pigment system facilitates organism response to both internal and external stimuli. Chromatic adaptation as well as color patterns are phenomena that, while occurring at the organism level, are also dependent on complex processes at the cell and molecular levels. Teleost fish are a group that probably presents the most diversified and complex pigment system of all living beings. Although a long-time topic of investigation, many of the themes dealt with aspects that require still wider analysis. This has only become possible through recent advances in science, and modern biological, genomics, and physiological cellular techniques.

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Chapter 8

Cellular and molecular features of skeletal muscle growth and plasticity

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Structural and morphological organization of skeletal muscle

Skeletal muscle is a complex and dynamic tissue, with remarkable morphological, metabolic, and functional adaptability to the most varied stimuli (Pette and Staron, 2001). Muscle tissue is built by highly specialized cells with differentiated morphology. These are the muscle fibers, which are elongated, cylindrical, and multinucleated, and have peripheral nuclei placed near the plasma membrane or sarcolemma (Fig. 8.1A). The muscle fiber diameter can vary from 10 to 100 μm and the length can reach from a few millimeters to several centimeters in vertebrates (Mescher, 2013; Listrat et al., 2016).

The sarcoplasm (cytoplasm) of muscle fibers shows transverse striations, also observed in light microscopy, corresponding to an alternation of dark bands (A bands) and light bands (I bands) (Fig. 8.1B). This transverse striation pattern is related to the presence of myofibrils, which fill almost all the sarcoplasm and are formed by a linear arrangement of sarcomeres, the contractile unit of the muscle fiber. The sarcomere is constituted by a set of different proteins, especially contractile, regulatory, and structural proteins (Huxley, 1969; Mescher, 2013).

In fish, 35%–60% of the total animal weight, i.e., most of the body mass, is represented by skeletal muscle (Listrat et al., 2016). This large proportion of muscle mass is important for several reasons. It ensures the generation of sufficient power required for rapid swimming (Bone, 1978), it is the main source of proteins to be used in energy-intensive activities (Weatherley and Gill, 1985), and it becomes commercially important because it represents the edible part of the fish.

Fish skeletal muscle differs from the other vertebrates by peculiar characteristics. In most fish, the skeletal muscle is segmented into morphofunctional units, the myomers, which repeat throughout the body and are separated by connective tissue, the myosepta, into which the muscle fibers are inserted (Alexander, 1969). The myosepta transmit the fiber contraction force from one myomer to another and to the axial skeleton and skin, with which it maintains structural continuity (Listrat et al., 2016). This arrangement in serial vertical units gives the fish greater mobility and efficiency during the wave and propulsion swimming movements (Van Leeuwen, 1999). Most fish have a connective tissue septum, the horizontal septum, that parts the muscle mass into epaxial (dorsal) and hypaxial (ventral) masses (Alexander, 1969; Greek-Walker and Pull, 1975) (Fig. 8.2).

In mammals, the skeletal muscle extracellular matrix plays an important role in muscle fiber force transmission, maintenance, and repair (Gillies and Lieber, 2012). These extracellular matrices show specific organization and consist of connective tissue organized in three sheaths: epimysium, which externally surrounds the muscle as a whole; perimysium, which divides the muscle into fiber fascicles; and endomysium, which surrounds each muscle fiber individually (Kjaer,

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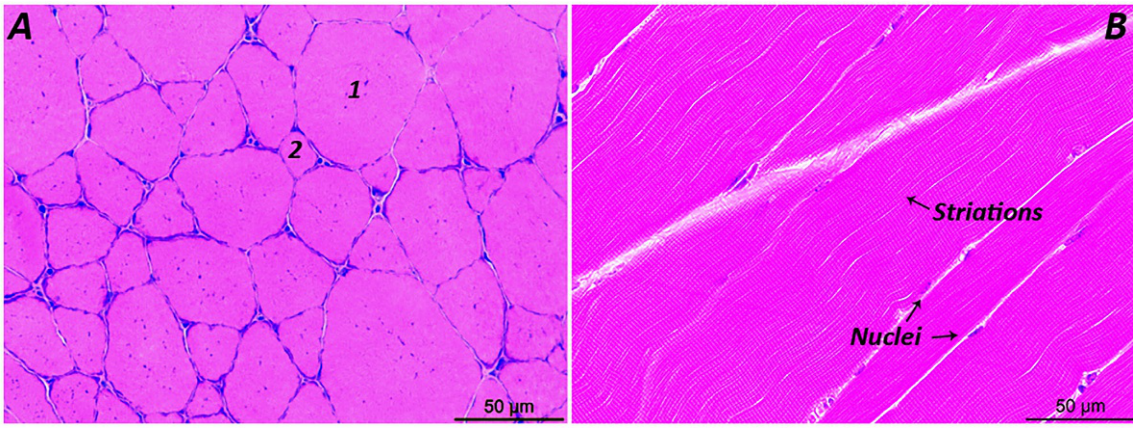

FIG. 8.1 (A) Transversal cross-section of the skeletal muscle of pacu (*Piaractus mesopotamicus*), showing round and/or polygonal muscle fibers with large (1) and small (2) diameters. (B) Longitudinal section of the skeletal muscle of pacu, showing transverse striations in the sarcoplasm represented by A (*dark*) and I (*clear*) bands. Peripheral nuclei (*arrow*). Hematoxilin and Eosin (HE). *(Images of own authorship.)*

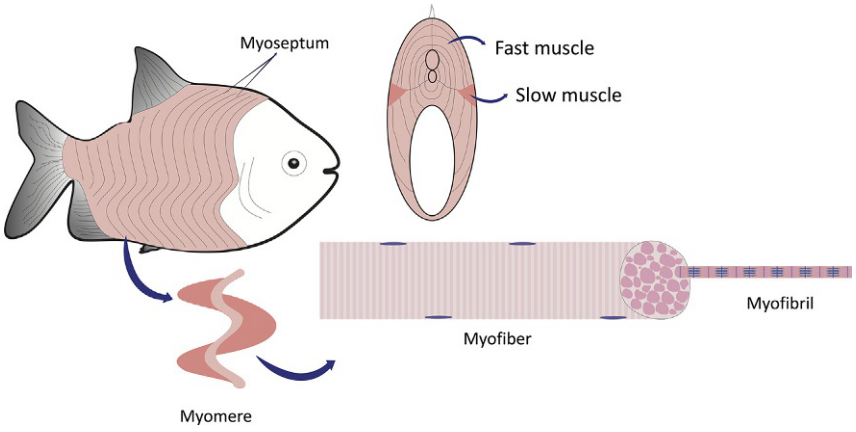

FIG. 8.2 Scheme of the anatomical organization of skeletal muscle in fish showing the myomere organization separated by the myoseptum; slow and fast muscle distribution; myofiber and myofibrils.

2004; Sanes, 2003). On the other hand, in fish skeletal muscle, the connective tissue distribution is specific for each species and the collagen concentration varies depending on the behavior of the animal, the activity performed during the swimming movements, and the growth stage (Ando et al., 1992; Carani et al., 2008; Michelin et al., 2009; Ofstad et al., 1996; Sato et al., 1986). In some fish, the connective tissue enveloping each muscle fiber is termed endomysium, and the thicker connective tissue that separates muscle fibers into fascicles is classified as perimysium, similarly to mammals (Carani et al., 2008; Michelin et al., 2009) (Fig. 8.3).

Mammalian skeletal muscles encompass different fiber types that differ according to their molecular characteristics as well as metabolic, structural, and contractile properties. Therefore, they can be grouped according to several parameters (Pette and Staron, 2001). The muscle fiber phenotype is first established during embryonic development by intrinsic mechanisms of myogenic control and is later modulated by neural and hormonal factors. The relative proportion of fiber types in any given muscle varies between species and individuals (Schiaffino and Reggiani, 2011).

Although different classifications have been proposed in the literature, the most informative methods to define muscle fiber types in mammals are based on the detection of the myosin heavy chain (MYHC) isoforms present in muscle fibers (Pette and Staron, 2000). This is done by using the myofibrillar ATPase (mATPase) histochemical technique, immunohistochemistry with specific antibodies for MYHC isoforms, and electrophoretic separation of MYHC isoforms in individualized microdissection fibers. These techniques revealed muscle fibers containing a single MYHC isoform, called pure fibers (I, IIA, IID/X, and IIB), and muscle fibers with two or more MYHC isoforms, referred to as hybrid muscle fibers (Pette and Staron, 2001). The contractile velocity of a muscle fiber is directly related to the type of MYHC expressed (for a in-depth review, see Talmadge et al., 1993). The MYHC capable of rapid hydrolysis of ATP characterizes type II fibers, which are fast-contracting fibers. In contrast, MYHC with slow ATP hydrolysis is found in slow-contracting type I fibers (Kelly and Rubinstein, 1994).

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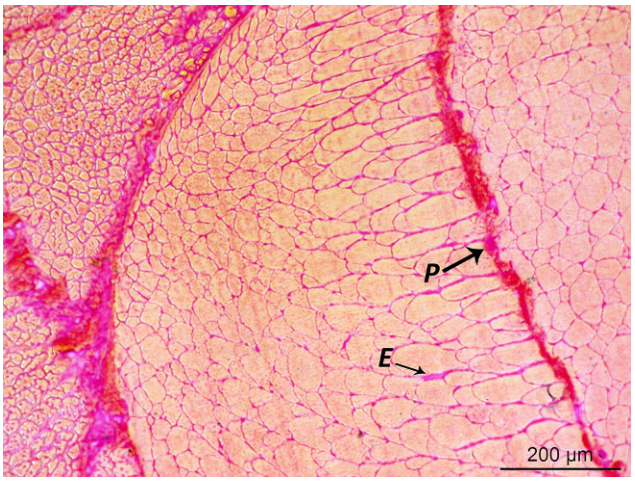

FIG. 8.3 Cross-section of the skeletal muscle of pacu. Perimysium (P) surrounding groups of muscle fibers. Endomysium (E) surrounding each muscle fiber. PicroSirius Red. *(Images of own authorship.)*

Fish skeletal muscle is characterized by three main muscle fiber types, identified and classified as slow, intermediate, and fast. Unlike mammals, each fiber type is located in anatomically separated compartments (Johnston, 1981a; van Raamsdonk et al., 1978, 1980). The slow fibers form the slow or superficial compartment, the fast fibers are distributed in the fast or deep compartment, and the intermediate fibers occupy the intermediate compartment located between the slow and fast compartments. This pattern of organization is described for most fish species, although variations may occur depending on the species and growth stage (Alexander, 1969; Weatherley and Gill, 1987).

Slow muscle fibers present the following characteristics: small diameter (25–45 µm), slow contraction, oxidative metabolism, many mitochondria and lipid droplets, high concentration of myoglobin, and well vascularized; these fibers are recruited during slow and sustained movements such as migration (Johnston, 1999). The slow compartment may appear as a thin uniform layer below the skin (Dal Pai-Silva et al., 1996) or present a more localized distribution near the lateral line nerve region, where it adopts a triangular aspect (Hoyle et al., 1986; Sänger and Stoiber, 2001). This compartment usually corresponds to less than 10% and never exceeds 30% of the entire muscle mass (Greek-Walker and Pull, 1975; Sänger and Stoiber, 2001). Fast muscle fibers are generally the largest in diameter (50–100 µm), have fast contraction and a glycolytic metabolism, few mitochondria and lipid droplets, a low concentration of myoglobin, and are less vascularized than slow fibers; these fibers are recruited during fast swimming movements, such as predation and escape behavior (Driedzic and Hochachka, 1976; Johnston, 1980). The fast compartment accounts for 70%–90% of the total muscle mass (Kilarski, 1990; Weatherley and Gill, 1989) and is important in aquaculture, as it is the core edible part of the fish (Zhang et al., 1996). Between the fast and slow compartments lies the intermediate compartment (intermediate musculature), reduced to one or two layers of cells that show fast contraction and an oxidative/glycolytic metabolism (Johnston, 1981b; Weatherley and Gill, 1987; Zhang et al., 1996) (Fig. 8.4).

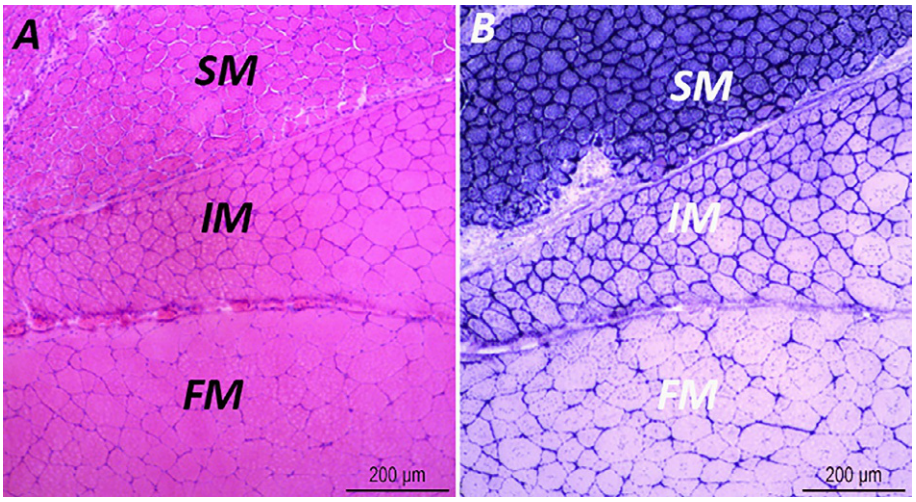

FIG. 8.4 Cross-section of the skeletal muscle of pacu showing the superficial slow muscle (SM), intermediate muscle (IM), and deep fast muscle (FM) compartments. (A) HE. (B) NADH-TR. *(Images of own authorship.)*

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Molecular control of skeletal muscle development

The embryonic development of skeletal muscle (myogenesis) arises from the segmentation of the paraxial mesoderm that originates at the somites. These units, located bilaterally to the notochord and the neural tube, are formed progressively along the rostral-to-caudal axis of the embryo (Kimmel et al., 1990). Each somite is subdivided into the sclerotome, myotome, and dermatome regions, each containing specific cell types (Currie and Ingham, 2001; Devoto et al., 1996). The sclerotome will give rise to the axial skeleton of the embryo while the dermatome will form the dermis and the myotome will originate the trunk and limb muscles (Currie and Ingham, 2001; Devoto et al., 1996). In fish, the sclerotome is reduced so most of the somite is made of the myotome, given that in the aquatic environment, the efforts of animals to sustain body weight are less demanding (Currie and Ingham, 2001).

The myogenesis of limbs and trunks in all vertebrates starts in the early embryo from somites (Comai and Tajbakhsh, 2014). General events of myogenesis involve the specification of the progenitor cells to form the myogenic precursor cell (MPC) lineage from the somites; these cells give rise to myoblasts that in turn differentiate into either slow or fast muscle fibers. In general, during myogenesis, MPCs differentiate into myoblasts, mononucleate cells presenting a few myofibrils, that merge with each other, giving rise to the multinucleated myotubes, with one or more central nuclei and peripheral myofibrils, which differentiate into muscle fibers (Blagden et al., 1997; Currie and Ingham, 2001; Ochi and Westerfield, 2007). In the myotubes, the nuclei migrate from the center to the periphery, setting off the organization of the proteins that make up the contractile unit of the muscle, the sarcomere; the myofibrils occupy almost all the sarcoplasm (Huxley, 1969; Johnston, 1999; Sänger et al., 1990; Sänger and Stoiber, 2001). At the same time, the development of the membrane system begins. This consists of T tubules and sarcoplasmic reticulum, both involved in the muscle contraction process, forming the triads. The myotube is then termed an adult muscle fiber (Flucher et al., 1992; Franzini-Armstrong, 1991; Kelly, 1971; Schiaffino and Margreth, 1969).

Myogenesis is a complex process that occurs by the actions of many signaling molecules temporally regulating myogenic cell lineage activation, proliferation, differentiation, and fusion to form the myotube that matures in the muscle fiber (Buckingham and Rigby, 2014). The MPCs are specified and determined by the activation of the paired-box transcription factor, the *pax3* gene (Bober et al., 1994; Relaix et al., 2004; Seale et al., 2000), under the action of extrinsic molecules such as the wingless/integrated protein family (WNTs) secreted from the neural tube and the surface ectoderm, the sonic hedgehog (SHH) secreted by the notochord, and the bone morphogenetic proteins (BMPs) secreted by the lateral mesoderm plate (Hernández-Hernández et al., 2017). In contrast to WNTs and the SHH signaling pathway, which actually have been demonstrated to induce the expression of the key transcriptional regulators of myogenesis, the myogenic regulatory factors (MRFs), the BMPs inhibit the expression of the MRFs (Hirsinger et al., 2001; Schuster-Gossler et al., 2007). The MRFs are a group of specific transcriptional factors including *myoD, myf5, myogenin (myoG),* and *mrf4* (Watabe, 1999; Weintraub, 1993) that act at multiple points in the myogenic program cooperatively, controlling myoblast determination, proliferation, and differentiation during embryogenesis and postnatal myogenesis (Buckingham and Rigby, 2014; Hernández-Hernández et al., 2017) (Fig. 8.5). MRFs share a highly conserved domain, the basic helix-loop-helix (bHLH), which displays 80% similarity in amino acid sequence (Edmondson and Olson, 1993). The bHLH domain of MRFs allows then to recognize the DNA consensus sequence known as E-box (5′-CANNTG-3′) in the regulatory sequences of the promoter region of most muscle-specific genes (Lassar et al., 1989; Murre et al., 1989; Blackwell and Weintraub, 1990). The helix-loop-helix region of MRFs is the binding domain of these molecules with the ubiquitously expressed E-proteins, such as E12 and E47, creating the MRF-E heterodimer (Murre et al., 1989). The binding of the MRF-E heterodimer or MRF homodimers to the E-box sequence activates the transcription and expression of muscle-specific genes (Lassar et al., 1991; Murre et al., 1989). *MyoD*, m*yf5,* and *mrf4* are directly related to the myogenic specification and control of myoblast proliferation while *myoG* and *mrf4* are imperative for myogenic differentiation during embryogenesis (Zammit, 2017). The loss of *myoD, myf5,* and *mrf4* results in a complete failure to specify myoblasts to form skeletal muscle, demonstrating a transcriptional network regulating the myogenesis (Rudnicki et al., 1993).

Although the myogenesis of teleosts shares many common characteristics with mammals, some features are unique to fish myogenesis (Barresi et al., 2001; Devoto et al., 1996, 2006). This process gives rise to distinct slow and fast twitch muscles that differ in contraction speeds, metabolic activities, and motoneuron innervation. For instance, the location and origin of these two different muscle types in zebrafish (*Danio rerio*) are topographically separable (Devoto et al., 1996, 2006). The commitment of the undifferentiated cells to embryonic myogenic progenitor cells that differentiate in myoblasts starts before the somitogenesis. In this phase, known as the primary or first wave of myogenesis, a subset of presomitic mesodermal cells, the adaxial cells, forms an epithelial-like monolayer flanking the notochord. The slow muscle fibers differentiation pathway is initiated in adaxial cells by the expression of *prdm1a* transcription factor activated by notochord-derived SHH protein (Baxendale et al., 2004; Roy et al., 2001). These cells start to express the myogenic

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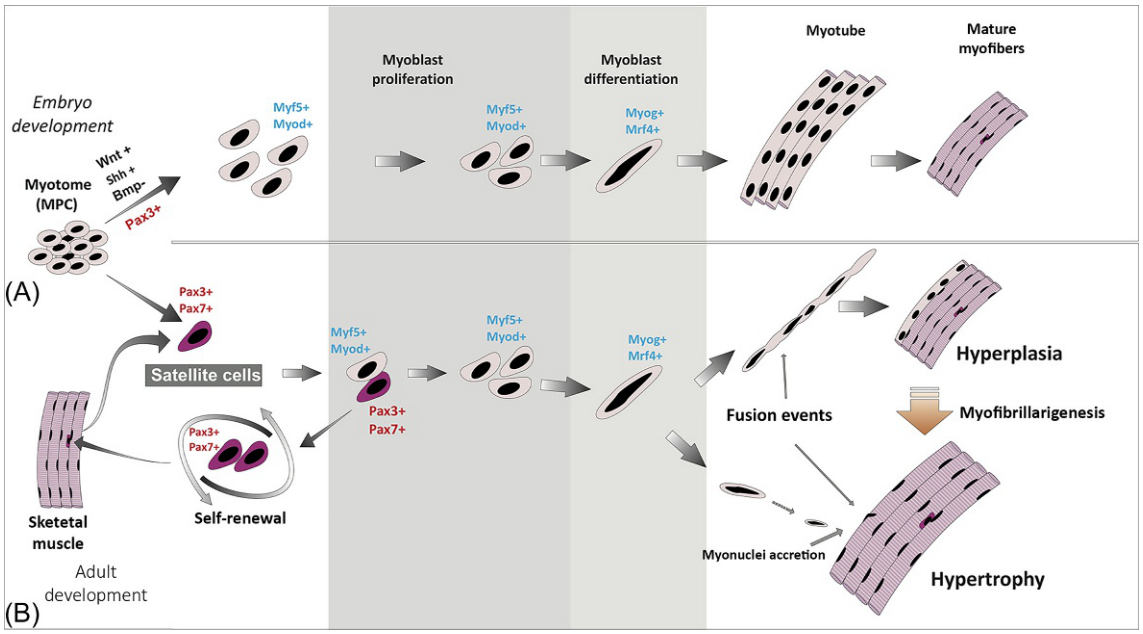

FIG. 8.5 (A) Scheme demonstrating the myogenesis process. Myogenic precursor cells (MPCs) lineage from the myotome compromised with the myogenic lineage express Pax3 that contributes to the expansion of myogenic precursor cells and differentiation of myoblasts. WNTs, SHH, and BMPs are involved with this process. MyoD and Myf5 control the myoblast proliferation. Myogenin and MRF4 control the myoblast differentiation into myotubes, which later differentiate to form mature myofibers. (B) Subpopulation of MPCs expressing Pax3 and Pax7 given rise to the satellite-like cells, located in the mature muscle fibers. These cells, when activated, proliferate under the control of MyoD and Myf5, differentiate under the control of Myogenin and MRF4, and control muscle growth by fiber hypertrophy (fibers increase in diameter and length with nucleus added in preexistent fiber) and fiber hyperplasia (increase of muscle fiber number). A population of satellite cells that expresses Pax3 and Pax7 are responsible for the self-renewal of satellite cells.

regulatory factors *myod* and *myf5* (Bentzinger et al., 2012; Devoto et al., 1996). After the somite formation, these adaxial cells migrate radially away from the notochord toward the surface of the myotome. They then differentiate, express *myog* and *mrf4,* and form a layer of cells on the superficial region, expressing *slow myhc* (Devoto et al., 1996).

Adaxial cells differentiate into slow muscle fibers to form the red (slow) muscle mass. After slow muscle fiber differentiation, the cells from the deeper and posterior part of the somites that do not migrate instead proliferate and differentiate into fast (white) muscle fibers, giving rise to the white (fast) muscle of the embryo (Currie and Ingham, 2001; Devoto et al., 1996; Ochi and Westerfield, 2007) (Fig. 8.6). These cells activate the transcription of fast specific genes, such as fast myosin heavy chain, as well as the transcription factor *sox6*, which downregulates the transcription of the slow specific genes, including *slow myosin heavy chain 1 (smynhc-1)* (von Hofsten et al., 2008). As suggested by von Hofsten et al. (2008) and Wang et al. (2011), the *sox6* gene plays a key role in fast muscle fiber differentiation by repressing the slow muscle fiber differentiation pathway in a *PR-domain-containing protein* (*prdm1a*)-dependent manner. The *prdm1a* is known as a repressor of the *sox6* transcription (Kinoshita et al., 2018; von Hofsten et al., 2008). After the primary

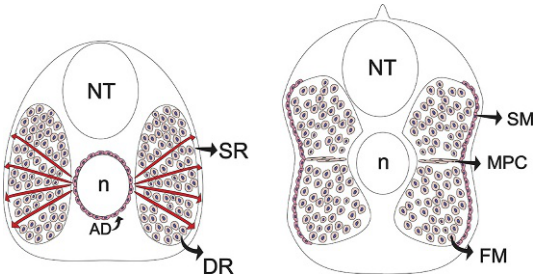

FIG. 8.6 Scheme of the differentiation of slow and fast muscles in fish. Adaxial cells (AD), epithelial-like monolayers flanking the notochord (n), migrate to the superficial region (SR) and differentiate into slow muscle (SM). The cells from the deeper and posterior part of the somites (DR) proliferate and differentiate into fast muscle (FM). A subpopulation of adaxial cells, referred as the slow muscle pioneer cells (MPC), emits cytoplasmic processes in the medial region, possibly to guide the migration of the adaxial cells toward the surface of the myotome. This medial region divides the myotome into the ventral (hypaxial) and dorsal (epaxial) regions. *NT*, neural tube.

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myogenesis, the secondary myogenesis occurs, contributing to the increase in size of the somite/myotome in a process named stratified hyperplasia, with the differentiation of new muscle fibers (Devoto et al., 1996; Elworthy et al., 2008). During stratified hyperplasia, dorsal and ventral germinal zones add new red and white muscle fibers in discrete layers of the myotome during muscle development (Rowlerson and Veggetti, 2001; Rowlerson et al., 1995).

A subpopulation of adaxial cells, referred to as the slow muscle pioneer muscle cells, emits cytoplasmic processes in the medial region that keep them connected to the notochord, possibly to guide the migration of the other adaxial cells toward the surface of the myotome (Currie and Ingham, 2001; Devoto et al., 1996; Du et al., 1997). In this medial region, this specialized structure forms the horizontal myossepta, which divides the myotome into the ventral (hypaxial) and dorsal (epaxial) regions (Johnston, 1981b) (Fig. 8.6).

Together with slow and fast muscle formation, a subpopulation of myogenic precursor cells does not fuse to form myotubes and takes a place near the myofibers late in the muscle development process. These cells express mainly the *paired box* 3 and 7 genes (*pax3* and *pax7*) and, in mice, this process occurs during fetal development (Kassar-Duchossoy et al., 2005). These cells give rise to the satellite cell population found in mammalian skeletal muscle (Dumont et al., 2015) or satellite-like cells in fish (Berberoglu et al., 2017; Hollway et al., 2007; Zhang and Anderson, 2014).

Molecular control of postembrionary skeletal muscle growth

Satellite cells or MPCs, which represent a population of about 2%–10% of the total myonuclei (Hawke and Garry, 2001; White et al., 2010), substantially contribute to muscle growth (Schultz, 1996). These cells are located close to the periphery of the muscle fiber in a specific position between the sarcolemma and the basal membrane (Mauro, 1961). Morphological characteristics of the satellite cells include a relatively high nuclear-to-cytoplasmic ratio, few organelles, and a smaller heterochromatic nucleus, as compared to the euchromatic myonuclei (Schultz and McCormick, 1994) (Fig. 8.7). The population of satellite cells is a reservoir of muscle progenitor cells that proliferates, differentiates, and contributes to muscle development (Relaix et al., 2005). Satellite cells are quiescent and transcriptionally less active in adult muscle, do not express the MRFs or other known markers of terminal differentiation, and are characterized by the expression of the *pax7* gene (Seale et al., 2000), shown to be important for the maintenance of satellite cells during perinatal and juvenile life (Rossi and Messina, 2014). Other well-known satellite cell markers have been identified and are restricted to either the quiescent, activated, or proliferative state or are expressed more widely, such as the cluster of differentiation 34 (Cd34; Beauchamp et al., 2000), M-caderin (Cornelison and Wold, 1997), C-Met receptor tyrosine kinase (c-Met; Cornelison and Wold, 1997), α7 Integrin (Gnocchi et al., 2009; Bockhold et al., 1998); Caveolin-1 (Cornelison and Wold, 1997),

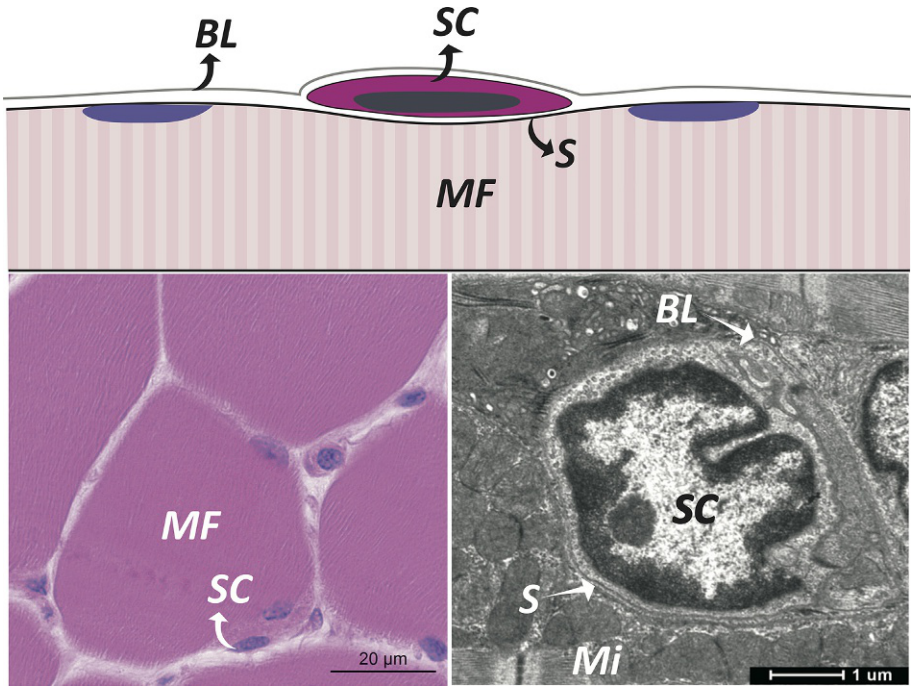

FIG. 8.7 (A) Scheme showing the satellite cell localization between the sarcolemma (S) of muscle fiber (MF) and the Basal Lamina (BL). (B) Photomicrography showing the location of the satellite cell (SC) at the periphery of the muscle fiber (MF) in a rat (*Rattus norvergicus*). (C) Rat satellite cell (SC) observed by electromycrograph. Sarcolemma (S); Basal Lamina (BL); Myofibrill (Mi). (B) HE; (C) Transmission electronic microscopy. *(Images of own authorship.)*

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Syndecan-3 and -4 (Cornelison et al., 2001, 2004), neural cell adhesion molecule (Ncam; Covault and Sanes, 1986); 5-Bromo-2′-deoxyuridine (BrdU; Schultz, 1996), and proliferating cell nuclear antigen (Pcna; Johnson and Allen, 1993).

Under growth stimulus or after injury, satellite cells become activated, proliferate, and exit from the cell cycle to generate myoblasts (Bischoff, 1986; Hurme and Kalimo, 1992; Zammit et al., 2002). These myoblasts downregulate the Pax7 expression, upregulate *myod*, *myog*, and *mrf4* expression. Besides this, myoblasts differentiate through fusion with each other, form de novo myotube to form newly myofibers or the myoblasts are internalized in preexisting muscle fiber (Fig. 8.5).

Unlike mammals that commonly show only fiber hypertrophy during the postnatal stage, the growth of fish muscle occurs by both hyperplasia and hypertrophy. Hyperplasia is defined as the production of new fibers, i.e., an increase in the number of muscle fibers, while hypertrophy is the increase in the diameter and length of fibers (Johnston et al., 2011; Rowlerson and Veggetti, 2001). The hyperplasia mechanism is separated in stratified and mosaic processes. The stratified hyperplasia occurs mainly in the larval stage and is similar to the embryonic stage, as the new fibers are produced in a growth zone, the germinal layer of the myotome (Rowlerson and Veggetti, 2001). In the mosaic, hyperplasia fibers originate in a disseminated way on the entire myotome, resulting in a mosaic pattern of muscle fiber diameter when skeletal muscle is observed in cross-sections. This process contributes to the fast growth in the juvenile phase, an interesting feature in aquaculture for fish that reach large sizes (Rowlerson and Veggetti, 2001) (Fig. 8.5).

The ability of fish that have undetermined growth to maintain postnatal hyperplasia seems to be related to the MPC action. The expression of *pax3* and *pax7*, in MPCs isolated from the fast muscle of the giant danio (*Devario* cf. *aequipinnatus*), showed a different pattern through myogenesis. While *pax7* expression decreased, the *pax3* expression increased during *in vitro* proliferation and differentiation, and this was correlated to the increased *myod* expression. The maintenance of *pax3* expression indicates a possible maintenance of the embryonic characteristic of giant danio MPCs, which continue being able to proliferate and, possibly, produce new muscle fibers resulting in muscle growth by hyperplasia (Froehlich et al., 2013a). In addition, when the number of MPCs was compared between fish families with undetermined and determined growth, larger species that had an undetermined growth pattern had a lesser proportion of MPCs than smaller ones. Therefore, the difference between these animals was related to the MPC sensibility to growth factors because larger fish had an expressive increase of MPC population after GH stimulation compared to their smaller relatives (Froehlich et al., 2013b).

An MPC role on skeletal muscle hyperplasia was proposed for Wuchang bream (*Megalobrama amblycephala*), which showed more MPCs surrounding the small fibers (hyperplastic process) than the larger fibers (hypertrophic process). Moreover, the higher number of bigger fibers in 2-year-old fish compared to 1-year-old fish showed the continuous recruitment of fibers through the life cycle, although this process was accentuated in younger animals as a result of the higher number of small fibers indicating hyperplasia (Zhu et al., 2014). This same contribution of hyperplasia and the hypertrophy process was characterized during pacu growth phases, in which a hyperplastic contribution was markedly higher in juveniles that presented a larger number of small fibers and a higher *myod* expression while hypertrophy was the process contributing the most to muscle growth in adult fish (De Almeida et al., 2008).

The activation of these processes is influenced also by extrinsic factors. Pacu hatched from eggs incubated at different temperatures had a varying hyperplasia and hypertrophy contribution for muscle growth. When eggs were incubated at 29°C, hatched fish presented a higher rate of cell proliferation and a great number of small fibers, indicating hyperplasia as the main process for muscle growth in these animals (De Assis et al., 2004). In another approach, refeeding of juvenile Nile tilapia (*Oreochromis niloticus*) subjected to fasting for five and 10 days induced fiber differentiation and proliferation, respectively, which was characterized by increased muscle fiber diameter and stimulated *myod* expression (Nebo et al., 2013).

Different contributions of hyperplasia and hypertrophy mechanisms for muscle growth were also reported for rainbow trout (*Oncorhynchus mykiss*) subjected to 60 days of food deprivation. After refeeding, there was an upregulation of genes related to protein synthesis, cell growth, and mitochondrial biogenesis while no alterations were registered for genes related to cell proliferation and DNA replication, processes that reflect hyperplasia, indicating a higher contribution of hypertrophy to muscle regrowth (Rescan et al., 2017). The findings herein discussed highlight not only the modulation of the hyperplasia and hypertrophy mechanisms considering fish species, but also the action of external factors as well as their contributions to muscle growth and maintenance in response to different stimuli.

Muscle growth is controlled by the coordinated action of different molecules involved in signaling pathways. Although differences between species are evidenced, their roles and mechanisms of action are generally well conserved among vertebrates (Nicoll et al., 2011). The hyperplastic and hypertrophic muscle growth mechanisms as well as the maintenance of the muscle phenotype are controlled positively and negatively by the activation or inhibition of several signaling pathways responsible for the gain (anabolism) and loss of muscle mass (catabolism) (Bonaldo and Sandri, 2013; Johnston et al., 2011).

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Muscle anabolism

The *igf-1-pi3k-akt-mtor* has been described as one of the major signaling pathways involved in muscle growth (Schiaffino et al., 2013). The interaction between the insulin-like growth factor I (*igf-1*) and its receptor leads to the stimulation of protein synthesis by the activation of phosphatidylinositol-3 kinase (*pi3k*), which promotes a membrane binding site for the serine-threonine kinase (*akt*). When active, this kinase starts the fosforilation of the mechanistic target of rapamycin (*mtor*) that stimulates protein synthesis by the activation of the p70S6K protein (Schiaffino et al., 2013). The *igf* system, constituted of *igf*s 1 and 2, their receptors, and *igf* binding proteins (*igfbp*s), is essential for formation, growth, and skeletal muscle maintenance (Funkenstein et al., 2006).

The *igf*-1 is involved in the regulation of the cellular metabolism of proteins, lipids, carbohydrates, and minerals as well as proliferation, cell differentiation, and body growth (Moriyama et al., 2000). Tissues and organs such as the brain, muscle, kidneys, and intestine locally produce *igf*-1, although the liver is the primary production site, providing approximately 75% of all the circulating *igf*-1 (Schwander et al., 1983). The importance of *igf*-1 for typical growth was demonstrated in knockout animal models. Knockout mice for *igf-1* showed severe retardation in growth, and animals that did not express the *igf-1* receptor did not survive after birth (Barton, 2006). However, in other experiments in which only *igf*-1 production by the liver was discontinued after birth, no body growth deficiency was registered (Sjögren et al., 1999). This demonstrated that the local production of *igf*-1 by other tissues and its autocrine or paracrine action is sufficient to ensure normal growth (Barton, 2006).

The *igf*-1 regulates the anabolic pathways of skeletal muscle, shaping the increase or maintenance of muscle mass (Otto and Patel, 2010). This increase in muscle mass (hypertrophy) may occur by the incorporation of satellite cell nuclei, after proliferation, in either growing or regenerating muscle fibers (Florini et al., 1994, 1996), and by the increase in protein levels within the muscle fiber (Otto and Patel, 2010). To trigger these actions, the *igf*-1 binding to its receptor may result in the activation of two main signaling pathways. First, *igf*-1 can stimulate the signaling cascade mediated by the mitogen-activated protein kinase (*mapk*), which results in myoblast proliferation during myogenesis, or it can stimulate satellite cell proliferation during muscle growth (Halevy and Cantley, 2004). The second signaling pathway that can be activated by *igf*-1 is mediated by the *pi3k*/*akt*/*mtor*, which determines the activation of different target proteins, resulting in an increase of the protein synthesis and hypertrophy in skeletal muscle (Lai et al., 2004). The maintenance of the muscular mass, mediated by *igf*-1, also occurs by the inhibition of the muscle atrophy pathways preventing the degeneration and loss of muscle mass (Otto and Patel, 2010).

In fish, the mechanisms involved with the signaling pathways activated by *igf*s (1 and 2) are still unclear, thus deserving of additional studies for a better understanding of their contribution in the muscle growth phenomenon. In zebrafish, *igf-1* stimulates the proliferation of myoblasts in cell culture through the activation of the *pi3k*/*akt* (Pozios et al., 2001). In a culture of rainbow trout myoblasts (Castillo et al., 2006) and salmon (Bower and Johnston, 2010a), the activation of the signaling pathways by *igf-1* was dependent on the development stage of these cells. In the proliferating and differentiating trout myoblasts, the *igf-1* activates the *mapk* and *pi3k*/*akt* pathways (Castillo et al., 2006). In pacu juveniles, *igf-1* and *igf-2* levels increased in fast muscle during approximately two days of fasting, with decreased expression levels 24 h after refeeding (Mareco et al., 2015). In salmon juveniles fasted for three weeks, Valente et al. (2012) registered a difference in *igf-1* and *igf-2* expression patterns, with the *igf-1* level increasing and *igf-2* decreasing after refeeding. Valenzuela et al. (2018) reported that key components of the *gh*/*igf* system, including ligands and receptors, were significantly downregulated during stress and concluded that chronic stress directly affects muscle growth and downregulates the *gh*/*igf* system.

Insulin b receptor knockout-zebrafish (*insrb* −/− fish) submitted to a high-carbohydrate diet had impaired growth hormone signaling, increased visceral adiposity, and fatty liver (phenotypes similar to the lipodystrophy observed in mammal) (Yang et al., 2018). In insulin a receptor knockout (*insra* −/− fish), the authors reported increased protein and decreased lipid content of the whole body. The authors concluded that through activation of the *gh* signaling pathway, *insrb* is more prone to promote lipid catabolism and protein synthesis, whereas lipid synthesis via glucose utilization is promoted primarily by *insra* action.

Studies on mammals have shown that the activation of *mtor*, a component of two different complexes, *mtorc1* (comprising *mtor*, *mlst8*, and *raptor*) and *mtorc2* (comprising *mtor, mlst8*, and *rictor*) (Sarbassov et al., 2006; Wullschleger et al., 2006), is a key regulator in the *igf* pathway to the maintenance and muscle mass gain (Bower et al., 2008; Johnston et al., 2011; Nobukuni et al., 2005; Sarbassov et al., 2005). Functional analysis in mice and human cell cultures showed a possible physiological activation of *mtorc1* in response to feeding, increasing the expression of genes encoding proteasome subunits, possibly related with protein turnover (Zhang et al., 2014). This fact promoted an increase in the intracellular pool of amino acids, thus influencing the rates of protein synthesis (Zhang et al., 2014). However, in fish, the knowledge of the signaling pathways regulating skeletal muscle mass, in particular the *mtorc* complexes, needs to be more explored. Paula et al. (2017)

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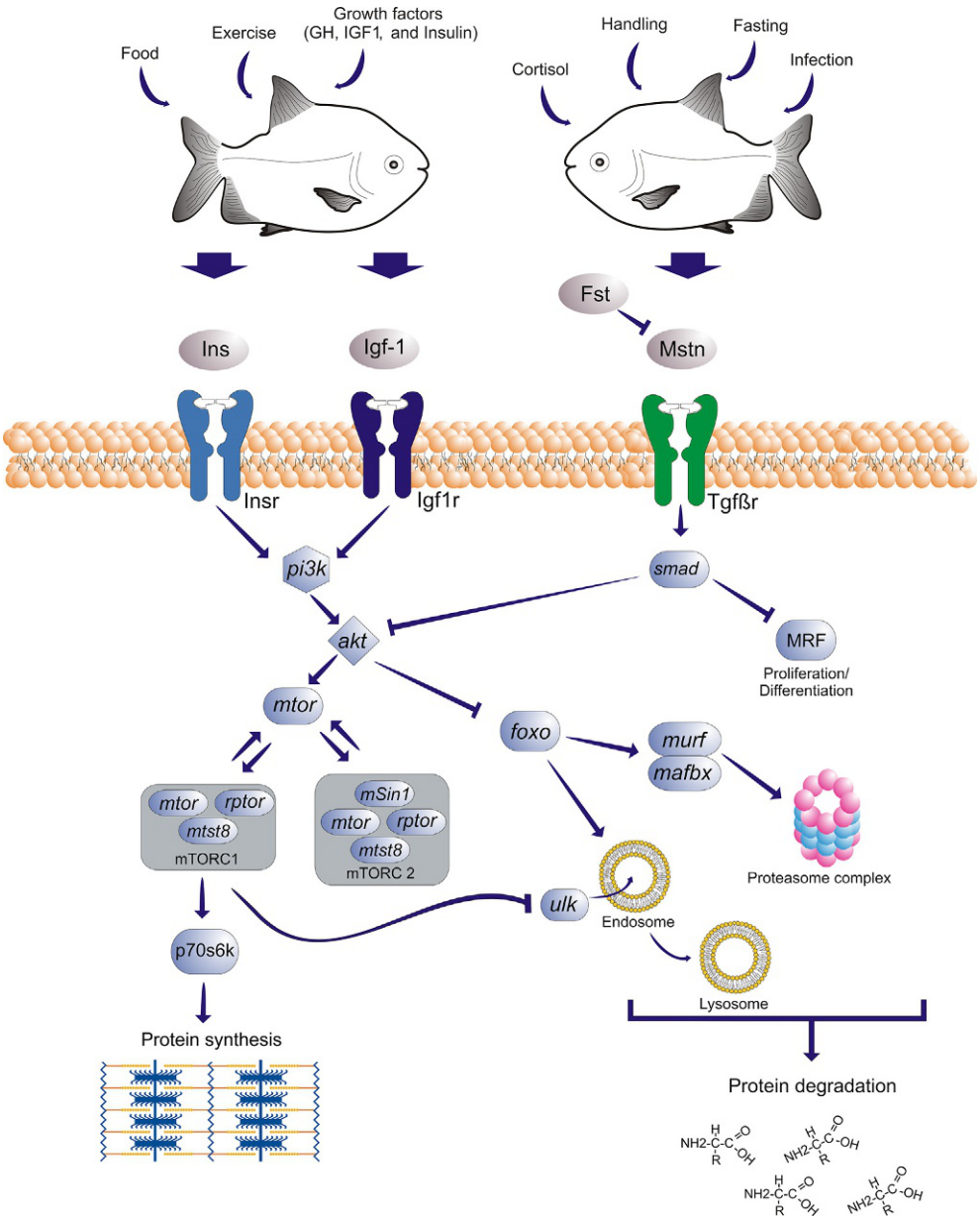

FIG. 8.8 Major pathways that control muscle fiber anabolism and catabolism. Protein synthesis and degradation are events regulated by different stimuli (anabolics and catabolics), which activate multiple signaling pathways, many of which converge at common intermediates and/or cross-talk with one another. See the main text for further details.

analyzed the *igf* system (*igf1*/*pi3k*/(*pik3c3*)/*akt*/*mtorc1*) of pacu juveniles and reported increased gene expression levels in fast and slow muscle after 10 days of food restriction, conversely to what had been described for mammals. The authors registered a high level of *mtorc1* complex (*mtor, mlst8,* and *raptor)* gene expression in both fast and slow muscles. The study actually demonstrated for the first time in fish that fasting conditions increased gene expression of the *mtorc1* complex in fast and slow muscles. Seiliez et al., 2011, 2013a) reported that in fish, the *akt*/*mtor* signaling axis was affected by the macronutrient composition of the diets after a single meal. The reduction of the dietary protein content in favor of carbohydrates impaired the activation of the *akt*/*mtor* signaling pathway. Finally, Azizi et al. (2016) demonstrated that lysine and leucine deficiencies downregulate the *igf* system and the *mtor* signaling pathway, subsiding the myogenesis process in gilthead sea bream (Fig. 8.8).

Muscle catabolism

Myostatin (*mstn*), also termed growth and differentiation factor-8 (*gdf-8*), is a member of a growth factor family known as transforming growth factor-β (*tgf-β*); it has been described as an inhibitor of muscle growth. In fish, Mstn and its receptor

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were detected in other tissues in addition to skeletal muscle (reviewed in Gabillard et al., 2013). This negative growth factor was first registered in mammals (McPherron et al., 1997) and, with genomic duplication, more than one copy was found in fish such as the pacu (Mareco et al., 2015), rainbow trout (Garikipati et al., 2006), and Atlantic salmon (Ostbye et al., 2001). In mammals, it is well established that the *mstn* interaction with proteins of the *smad* family that, when bond with their receptors, translocate to the nucleus and control the transcription of a number of genes, such as the *mrf*s (reviewed in Kollias and McDermott, 2008). Moreover, *mstn* also regulates the cell cycle progress by stimulating p21 expression, an inhibitory protein related to the maintenance of the cells in the gap phases, resulting in decreased cell proliferation (Thomas et al., 2000).

Postnatal muscle growth and myogenesis are controlled by *mstn*, which reduces the expression of components that participate of the *igf-1* pathway, as seen in rainbow trout myotubes treated with human recombinant *mstn* (Seiliez et al., 2013b). Moreover, despite the unspecific action of *mstn* in other tissues than skeletal muscle (Gabillard et al., 2013), this negative control was also found in Nile tilapia fast muscle, which had a decreased fiber diameter and increased *mstn* gene expression during fasting (Nebo et al., 2013). In accordance with these observations, the overexpression of Follistatin in rainbow trout, an antagonist that strongly binds to *mstn* (Amthor et al., 2004), resulted in muscle growth mainly by hyperplasia (Medeiros et al., 2009) while in *mstn*-mutaded medaka (*Oryzias latipes*), it occurred mainly by hypertrophy (Yeh et al., 2017). On the other hand, no differences or minimal alterations in this gene expression were found in pacu skeletal muscle under different temperatures (Paula et al., 2014). These results show the impacts of external and internal conditions on the *mstn* expression that also vary according to fish species.

Besides the negative action of *mstn* on muscle growth, the regular maintenance of skeletal muscle is also controlled by protein catabolism, an important process for many organisms that is essential for muscle turnover. Regarding mammals, the amino acids obtained from muscle degradation act as an energy source for others tissues in situations such as cancer, AIDS, and heart failure (Bonaldo and Sandri, 2013); for fish, the muscle role as an amino acid source is significant in periods of fasting and gonad maturation (Johnston et al., 2011). However, the exacerbated catabolism process results in tissue atrophy, a situation defined as the decrease of the cell volume resulting from degradation of its components, which can hamper animal growth (Bonaldo and Sandri, 2013). Two main systems are involved in proteolysis: the proteasome and the autophagy machinery (Yorimitsu and Klionsky, 2005).

Autophagy is a process of "self-eating" that allows cellular adaptation to nutritional and energetic changes. It initiates with the engulfment of the degradation target and ends with the digestion on the lysosome (Kim et al., 2011; Yorimitsu and Klionsky, 2005). Seiliez et al. (2014) demonstrated the importance of this system in fish skeletal muscle using rainbow trout myotubes subjected to serum deprivation, a condition that simulates muscle catabolism *in vitro*, and in which autophagy was responsible for 40%–49% of total protein degradation. Moreover, a decrease in the expression of unc-51-like autophagy activating kinase 1 (*ulk1*), a kinase related to the autophagy initiation and controlled by the *torc1* complex (Kim et al., 2011), was registered for the skeletal muscle of pacus subjected to food restriction, possibly because of an inhibition by *mtor* (Paula et al., 2017). In the same study, the *bcl2*, an antiautophagic protein that circumvents this process by interaction with a proapoptotic and proautophagic molecule (Pattingre et al., 2005), had an increase on its expression as a possible way to reduce exacerbated muscle loss in such a condition. Besides these factors, lysosomal protease members of the Cathepsins (*cts*) family and nonlysosomal protease members of the Calpains (*capn*) family are known to be involved in *postmortem* changes of meat texture, possibly as a result of myofibrillar protein proteolysis (Nagaraj and Santhanam, 2006). The *capn* participation in the regulation of skeletal muscle is exemplified in gilthead sea bream (*Sparus aurata*) subjected to fasting, which showed an increase in the *capns1b* expression during restriction and a decrease in *capns1a* expression with the increase of carbohydrate percentage in the meal. These finds highlight, respectively, an increase in protein degradation and a possible regulation of these genes according to diet composition (Salmerón et al., 2013). Similarly, there was an increase of *capn* and *cts* protein levels in the skeletal muscle of gilthead sea bream subjected to sustained and moderate exercise, showing a role of these molecules in muscle remodeling, an important process for muscle growth (Vélez et al., 2017).

In addition to the autophagy mechanism, a more specific system known as ubiquitin-proteasome (UPS) also contributes to muscle wasting. The mode of action of such a system involves enzymes essential to target ubiquitination. This process initiates with the action of E1 enzymes that activate the ubiquitin proteins. The ubiquitin is then transferred to E2 enzymes, and E3 enzymes catalyze the binding of the ubiquitin protein to the target. After ubiquitination, the substrate is degraded on the proteasome 26S (reviewed in Bonaldo and Sandri, 2013). Members of a transcription factors family known as *foxo* also participate in the degradation process. When translocated to the nucleus, *foxo3* activates the transcription of the E3 class enzymes, such as the atrogenes *mafbx* (or *atrogin-1*) and *murf1* (Sandri et al., 2004). Although an *in vitro* approach indicates that the UPS does not have as intense a role in muscle catabolism as the autophagy-lysosomal system (Seiliez et al., 2014), a number of situations that led to fish skeletal muscle loss were related to an increase of these atrogenes. The major part of

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these studies focused on situations of physiological stress that occur under intensive farming conditions, such as handling stress, food restriction, and microorganism infections. An increase in atrogene expression was found in the skeletal muscle of Atlantic salmon subjected to seven days of fasting while a posterior decrease was seen with a continuous refeeding (Bower and Johnston, 2010b) and in response to a single meal (Valente et al., 2012). A period of 28 days of starvation caused the same effect and, in addition, increased the expression of the proteasome catalytic subunit (Tacchi et al., 2010). By using a different approach, when injected with LPS (a bacterial mimic that induces an antibacterial response) the skeletal muscle of Atlantic salmon had an increase in atrogene expression, indicating a response of this system to a possible energy reallocation resulting from bacterial infection (Tacchi et al., 2010). Moreover, a similar result was found in the primary culture of Atlantic salmon skeletal muscle in response to the immune modulators *interleukin-1β* and *interferon* type-1, showing a relationship between the inflammation and catabolic pathways to reallocate nutrients in this situation (Heidari et al., 2015). *In vitro* (cortisol supplementation) and *in vivo* (handling) stress situations also led to increased atrogene expression in the skeletal muscle of the red cusk-eel (*Genypterus chilensis*) with, in the *in vitro* experiment, a decrease in the diameter of the myotubes (Aedo et al., 2015). In rainbow trout, an increase in atrogene expression in response to food restriction was also found with a posterior decrease after *igf-1* treatment (Cleveland et al., 2009). The protocol of fasting and refeeding in the skeletal muscle of pacus also caused an atrogene increase and decrease, respectively, and allowed the analysis of ubiquitin-specific protease (USPs) expression patterns (Mareco et al., 2015). In this same species, fasting for 10 days led to increased atrogene expression in fast and slow muscles, with decreased expression of these molecules during refeeding (Paula et al., 2017).

Altogether, these works show the importance of the pathways that control the muscle mass, either as providing energy in unfavorable conditions or avoiding exacerbated muscle growth. Moreover, the intrinsic relationship of the pathways involved in the growth and degradation of skeletal muscle is essential for muscle turnover and maintenance (Fig. 8.8).

MicroRNAs regulating skeletal muscle growth and phenotype

In the late 1940s and 1950s, DNA was described as the carrier of genetic information for the synthesis of proteins, the molecules responsible for most cellular functions. The central dogma of molecular biology was defined, with DNA promoting synthesis of RNA that would thus be translated into protein. Currently, the core of knowledge on the propagation of genetic information is understandably much more complex with several other mechanisms involved in the control of cellular physiology and the definition of cell phenotype, such as DNA methylation, histone modification, and regulation by noncoding RNAs (Chuang and Jones, 2007).

Among the noncoding regulatory RNAs, the microRNAs (miRNAs) stand out. They are a class of small RNAs that plays several important roles in most biological processes, including skeletal muscle development and growth (Bizuayehu and Babiak, 2014; Ge and Chen, 2011). The main function of miRNAs is posttranscriptional gene regulation, promoted by the translation inhibition or decay of messenger RNAs (mRNAs) (Bartel and Chen, 2004; Filipowicz et al., 2008; Ge and Chen, 2011). This repression occurs through base complementarity between nucleotides 2 ando 8 of the 5′ region of the miRNA, known as the seed sequence, and the 3′ untranslated region (3′ UTR) of the target mRNA (Williams et al., 2009) (Fig. 8.9).

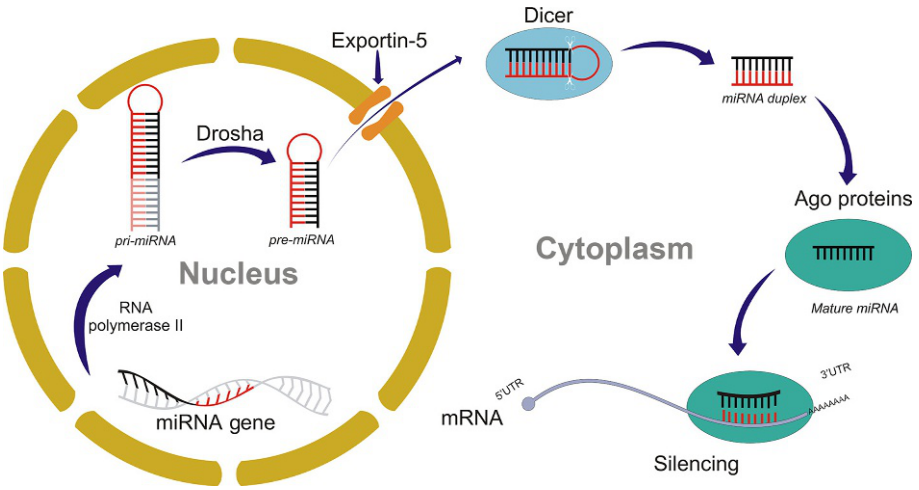

FIG. 8.9 Nucleotide complementarity between miRNA and target mRNA. Complementarity usually occurs through binding between nucleotides 2–8 of the 5′ region of the miRNA (seed sequence) and the 3′ UTR of the target mRNA. This binding is determinant in target recognition and sufficient to trigger silencing. *ORF*, open reading frame; 3′ UTR (3′ untranslated region).

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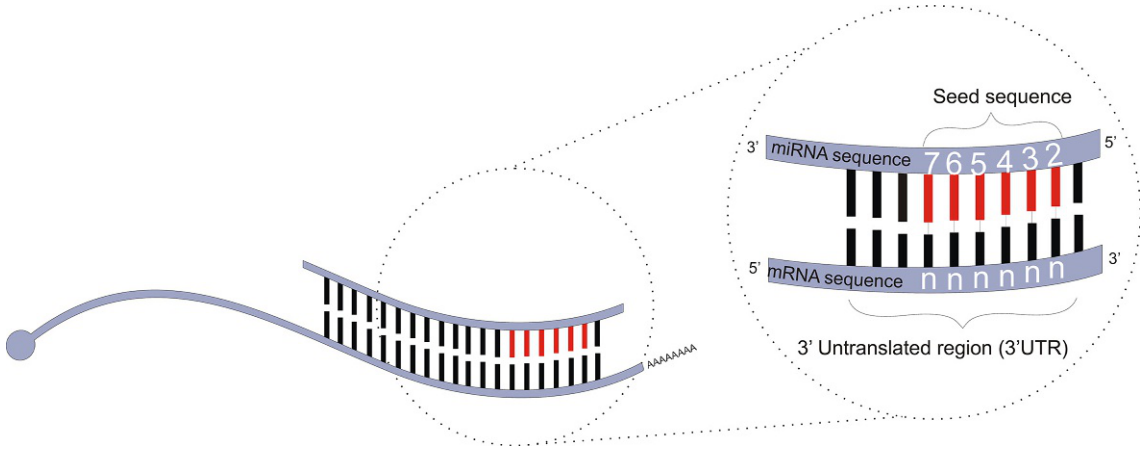

FIG. 8.10 miRNA biogenesis. The miRNA regulatory pathway begins with gene transcription into pri-miRNA and its processing into pre-miRNA, within the nucleus. The pre-miRNA is transported to the cytoplasm and cleaved to produce a double-stranded miRNA. The functional strand is incorporated into a silencing complex, which will bind to the target mRNA and promote its degradation or translational repression.

MiRNA genes are synthesized in the nucleus as long primary transcripts, known as pri-miRNAs, mainly by RNA polymerase II. The pri-miRNAs can encode one or more miRNAs, and are further processed by the RNAse III enzyme Drosha (Lee et al., 2003) to hairpin structures with approximately 70 nucleotides, known as precursor miRNAs (pre-miRNAs). Pre-miRNAs are then transported by Exportin-5 to the cytoplasm, where they will be cleaved by the RNAse III enzyme Dicer into a double-stranded miRNA (duplex) with approximately 22 nucleotides (Ge and Chen, 2011; Winter et al., 2009). Thereafter, only a single strand of the miRNA duplex is incorporated to the Argonaut family (AGO) proteins to form the RNA-induced silencing complex (RISC), which associates with the target mRNA complementarity site to promote its silencing (Bizuayehu and Babiak, 2014; Winter et al., 2009). The other duplex strand is degraded or may associate with a new RISC to regulate another target mRNA (Fig. 8.10).

MiRNAs regulate their targets in a combinatory pattern, increasing the complexity and regulatory potential of gene expression. A single miRNA can target several mRNAs and, similarly, individual mRNAs can be targeted by different miRNAs (Guo et al., 2010; van Rooij et al., 2008). Thus, most miRNAs are able to fine-tune the regulation of signaling pathways and common biological functions.

Phylogenetic analysis reveals a high miRNA conservation among vertebrates, suggesting a positive correlation between the number of miRNA genes and the complexity of the organism (Lee et al., 2007; Sempere et al., 2006). Moreover, studies show that more than 60% of mRNAs in mammals have conserved miRNA-binding sites (Friedman et al., 2009; Guo et al., 2010). Based on this fact, it can be inferred that a substantial set of mRNAs is modulated by miRNAs also in teleost fish (Bizuayehu and Babiak, 2014). In such a context, the extent of miRNA participation in the regulatory network in teleost fish could be even higher and more complex, due to an event of whole genome duplication (WGD). Around 450–320 million years ago, teleost fish underwent a specific WGD (teleost-specific genome duplication, TSGD) (Jaillon et al., 2004), which promoted a differential retention of duplicated genes (Garcia de la serrana et al., 2014). This TSGD allowed the expansion of components in teleost molecular networks compared to other vertebrates, including the miRNAs (Berthelot et al., 2014).

MiRNAs in teleost fish are involved in embryogenesis and several developmental and physiological processes in different tissues (Biyashev et al., 2012; Flynt et al., 2009; Giraldez et al., 2006; Huang et al., 2012; Staton et al., 2011; Wienholds et al., 2005; Yan et al., 2012b; Yin et al., 2008, 2012). Both muscle development and growth are deeply regulated by miRNAs; some of them are classified as miRNA muscle-specific due to their specific or high expression in cardiac and/or skeletal muscles, notably the miR-1, miR-133, miR-206, and miR-499. These miRNAs are involved in myogenesis, myoblast proliferation and differentiation, fiber type specification, and muscle regeneration (Chen et al., 2006, 2010; Ge and Chen, 2011; McCarthy, 2011; van Rooij et al., 2009).

Both miR-1 and miR-133 influence the gene expression patterns during muscle embryogenesis in zebrafish (*Danio rerio*), regulating sarcomeric actin organization (Mishima et al., 2009). A possible stimulatory role of miR-206 in myoblast differentiation was shown by Duran et al. (2015) in both the fast- and slow-twitch muscles of pacu (*Piaractus mesopotamicus*). The authors observed an inverse correlation between the expression levels of miR-206 and *pax7* mRNA, a transcription factor required for the survival of myogenic precursor cells (MPCs) and subsequent myoblast proliferation. In addition, Yan et al. (2013) showed in Nile tilapia that miR-203b directly regulates *myod*, an essential myogenic regulatory factor that modulates myoblast activation, proliferation, and differentiation during myogenesis. Molecular analyses

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revealed that miR-1, miR-133, and miR-206 expression levels increased according to the progression of skeletal muscle growth in different development stages of pacu (Duran et al., 2015), and the same miRNAs had increased expression also during muscle development of Nile tilapia (*Oreochromis niloticus*) (Nachtigall et al., 2015; Yan et al., 2012a) and common carp (*Cyprinus carpio*) (Yan et al., 2012c). Huang et al. (2012) also identified a differential expression pattern of miRNAs involved in *gh*/*igf1* signaling in the skeletal muscle of fast-growing Nile tilapia that may serve as molecular markers in fish breeding programs. Moreover, several miRNAs were reported as regulating the changes from the hyperplasic to the hypertrophic phenotype in the skeletal muscle of zebrafish, providing evidence for the involvement of miRNAs in muscle growth transitions (Johnston et al., 2009).

MiRNAs are also involved in fiber type specification and muscle phenotype determination, especially miR-499. Wang et al. (2011) showed that miR-499 mediates translational repression of *sox6* in the slow-twitch fibers of zebrafish, results corroborated by analyses in Nile tilapia (Nachtigall et al., 2015). The sox6 is a transcriptional activator that plays a key role in the maintenance of the fast-twitch phenotype, repressing slow-twitch-specific genes such as slow myosin heavy chain 1 and slow troponin (von Hofsten et al., 2008). Duran et al. (2015) observed increased expression of miR-499 in the slow-twitch muscles of juvenile and adult pacus, whereas *sox6* transcripts had higher expression in fast-twitch fibers at both development stages. In addition, the increasing expression of *sox6* accompanied the growth of fast-twitch muscle, as a result of the decreased levels of miR-499 as the fibers matured and differentiated (Duran et al., 2015). Altogether, these studies indicate the involvement of miR-499 in the specification and maintenance of the slow-twitch phenotype in muscle fibers, a mechanism conserved among vertebrates (Wang et al., 2011).

Primary myoblast cell culture as an in vitro tool to study myogenesis

Cell culture is an *in vitro* technique that allows the maintenance and study of cells outside the body of the original organism. Although the C2C12 cell line is widely used to understand the regulation of muscle growth and development in mammals (Yaffe and Saxel, 1977), it is inadequate and not recommended for the transfer of information from these cell cultures to fish species because of differences in fiber type organization and molecular networks regulating muscle growth. Besides, muscle cell lines from teleost fish have not yet been obtained, making necessary the establishment of primary cell cultures of myoblasts to study fish skeletal muscle (Johnston et al., 2011). Primary cell cultures are those prepared directly from the tissue of interest that have the same cellular characteristics, constituting a procedure that holds better similarity to *in vivo* studies. However, the primary cell cultures assays must be performed quickly because the cells can maintain their original properties only for a short period of time before entering apoptosis (Freshney, 2010).

Fish myoblast cell cultures encompass the main stages of myogenesis, with initial quiescent myogenic precursor cells and early myoblast commitment, myoblast proliferation and migration, differentiation and fusion into myotubes, and maturation to muscle fibers (Gabillard et al., 2010b; Vélez et al., 2016) (Fig. 8.5). Moreover, the cell culture system provides an *in vitro* environment with fewer variables than those found in skeletal muscle *in vivo*, allowing the analysis of many signaling pathways under controlled conditions. This enables an in-depth study of regulatory molecules and the investigation of their roles at different stages of cell culture (Froehlich et al., 2013b; Gabillard et al., 2010a), especially through the gain and loss of function assays (Johnston et al., 2011). Furthermore, myoblast cell culture media can be modified to access the role of nutrients, growth factors, hormones, drugs, and temperature in regulating the muscle growth process (Bower and Johnston, 2010a; Castillo et al., 2004; Gabillard et al., 2010a; Garcia de la Serrana and Johnston, 2013; Seiliez et al., 2012; Vélez et al., 2014) as well as many other manipulative and pharmacological assays (Fig. 8.11).

Froehlich et al. (2013b) evaluated molecular markers of *in vivo* fish myogenesis in myoblast cell cultures of the giant danio (*Devario aequipinnatus*). They reported that while *pax7* and *myf5* are expressed chiefly in newly activated myogenic precursor cells, and *myog* is expressed mainly in myotubes, *pax3*, *myod,* and *mstn* expression occurs throughout all the cell culture stages (Froehlich et al., 2013b). Atlantic salmon (*Salmo salar*) muscle cells were used to determine the mRNA expression of *myod* paralogues during cell culture development, with *myod1b* and *myod1c* more related to proliferation and *myod1a* more related to differentiation, proving the existence of a *myod* subfunctionalization mechanism after the WGD (Bower and Johnston, 2010c). An experiment performed by Gabillard et al. (2010a) produced transgenic rainbow trout (*Oncorhynchus mykiss*) carrying a green fluorescent protein (GFP) under control of a fast myosin light chain promoter to monitor in vitro myoblast differentiation. Myoblasts extracted from transgenic fish expressed GFP once myoblasts started to fuse and differentiate during myotube formation, consolidating an *in vitro* real-time monitoring system that offered numerous applications in fish muscle growth studies. Besides, the authors showed that temperature, a crucial environmental factor for muscle growth, directly influenced the differentiation and fusion of myogenic cells (Gabillard et al., 2010a).

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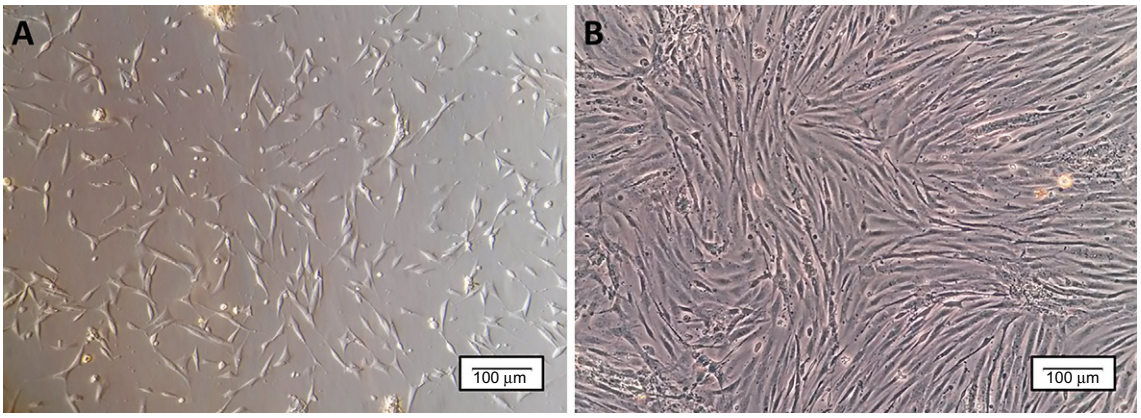

FIG. 8.11 Primary myoblast cell cultures established from the fast-twitch skeletal muscle of juvenile pacus. (A) Proliferative myoblasts at day 3 of cell culture. (B) Myoblast differentiation and fusion into myotubes at day 8 of cell culture. The images were obtained on an inverted microscope (10 × magnification; Bars: 100 µm). *(Images of own authorship.)*

Several studies used fish myoblast cell cultures to examine anabolic pathways related to muscle growth, especially *insulin*, *igf,* and *mtor* signaling (Bower and Johnston, 2010a; Castillo et al., 2006; Cleveland and Weber, 2010; Díaz et al., 2009; Seiliez et al., 2008; Vélez et al., 2014). Díaz et al. (2009) detected a gradual increase of *glut4* expression throughout differentiation of rainbow trout muscle cells, and both myoblasts and myotubes had *glut4* upregulation after treatment with *insulin* and *igf1*. Moreover, *igf1* and *insulin* supplementation to trout myocytes promoted increased protein synthesis (13% for both growth factors) and decreased proteolysis (14% and 17%, respectively), whereas leucine administration only reduced protein degradation (8%) (Cleveland and Weber, 2010). The synergistic effect of combining growth factor treatments is also commonly evaluated in fish myoblast cell culture studies. In fact, the combination of *igf1* and amino acid treatments in the starved muscle cells of Atlantic salmon led to a huge increase of *igf1* transcripts, indicating a possible positive feedback mechanism of *igf1* production (Bower and Johnston, 2010a). Moreover, some previous works showed that igf-induced protein synthesis occurs only if amino acids are also present in cell culture media (Bower and Johnston, 2010a; Garcia de la Serrana and Johnston, 2013), whereas amino acids alone are able to stimulate protein synthesis signaling in fish myoblast cell cultures (Seiliez et al., 2008; Vélez et al., 2014).

The role of nutrition on the modulation of skeletal muscle growth was also evaluated in a recent study performed by the authors of this chapter. Because it is unclear if ascorbic acid can directly regulate myogenesis, besides its action on connective and bone tissues during fish growth, an evaluated trial was carried out to evaluate the effect of ascorbic acid supplementation in the controlled environment of pacu myoblast cell cultures (Duran et al., 2019). Ascorbic acid enhanced myoblast proliferation and migration, in part due to its antioxidant role. In addition, myoblasts supplemented with this vitamin had increased *myod and mtor* expression, important molecular markers of myogenesis and protein synthesis, respectively.

Myogenesis and muscle growth regulation by muscle-specific miRNAs were also accessed using an *in vitro* system. Duran et al. (2015) used pacu myoblast cell cultures to complement the *in vivo* analysis of miR-1, miR-133, and miR-206 in both fast- and slow-twitch muscles. As in muscle samples, the cell cultures also showed an inverse correlation between miRNAs and their target expression, corroborating miR-1 and miR-206 roles in myoblast differentiation. However, *in vitro* analyses allowed further insight into how miR-133a and miR-133b regulate myoblast proliferation and differentiation, proving their use as a way to expand our knowledge on the molecular mechanisms that regulate muscle growth (Duran et al., 2015; Johnston et al., 2011).

Another key aspect comes from the compartmentalization of skeletal muscle in fish, which allows the establishment of individual myoblast cell cultures from fast- and slow-twitch muscles. Certain processes, for instance development, metabolism, and fiber type determination, are quite different according to muscle type, and such studies could be addressed in those different myoblast cell cultures. This separate study of muscle types is practically impossible in mammals, given the mosaic pattern of skeletal muscle with fast fibers located between slow fibers (Schiaffino and Reggiani, 2011), making fish ideal models for this type of research. For this purpose, it is necessary to use fish that exhibit indeterminate somatic growth, which leads to the formation of new fibers for a prolonged period of time and provides a great increase of muscle mass (Rowlerson and Veggetti, 2001). Small fish, such as zebrafish, make almost impossible the isolation of enough myoblasts from both fast and slow muscles, which strongly restrains any myoblast cell culture study (Froehlich et al., 2013b). Works published so far had only developed fish myoblast cell cultures for fast-twitch muscle, but a slow myoblast cell culture from

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rainbow trout was successfully developed by Duran et al. (2019). Despite no morphological differences between slow and fast myoblasts, gene expression revealed significant alterations in markers of contractile properties (*slow myhc* and *fast myhc*), fiber type specification (*six1*, *sox6,* and miR-499 paralogues), and metabolism (*glut4* and *pgc1α*). The slow muscle in vitro system generates a very useful tool to future comparative studies between fast- and slow-twitch muscle development and regulation, both in fish and in mammals.

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Chapter 9

The cardiovascular system

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Chapter outline

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Introduction

The teleost fish constitute the group of vertebrates with the largest number of species, dominating both marine and freshwater environments (Nelson, 1994). About 30,000 species of fish occupy a wide variety of habitats that differ widely with respect to abiotic factors such as temperature, dissolved oxygen, pH, depth, and luminosity, and biotic conditions such as food availability, predation, and competition (Wootton, 1990).

Fish are exposed to large seasonal and/or acute variations in temperature (Farrell, 1984). Thus, the temperature-dependent regulation of cardiovascular function is of major importance for these animals. For example, the vertical movement of fish in the water column can cause significant and rapid oscillations in body temperature. Because these changes may be unpredictable, fish hearts must have some intrinsic resistance to severe changes in temperature. In addition to the acute changes, many species of teleost fish are also subject to large seasonal variations of temperature, which are usually more regular and predictable. Thus, the ability of the heart to acclimatize is of particular importance for fish in regions susceptible to large seasonal changes in environmental temperature.

Acute and seasonal changes in the water oxygen content also have a great impact on the energy available to the fish, being able to determine their levels of activity and, therefore, the demands on the cardiac function (Driedzic and Gesser, 1994). Depending on the current environmental factors and their fluctuations in different habitats, fish show a variety of lifestyles and activity levels, which are reflected in the structure and function of the cardiovascular system (Santer, 1985; Farrell and Jones, 1992).

Adaptation to a wide variety of habitats, lifestyles, and activity levels has resulted, throughout evolution, in an impressive interspecific variation in both the anatomy and physiology of the fish cardiovascular system.

This chapter aims to describe the anatomy of the cardiovascular system of teleost fish and discuss the main mechanisms of functioning and adaptation to the environment.

Heart anatomy

The teleost fish heart is a four-chambered organ surrounded by a relatively rigid pericardial sac located in the ventral position immediately behind the gills. The four chambers—sinus venosus, atrium, ventricle, and bulbus arteriosus—are arranged in series and pump deoxygenated venous blood to the gill circulation, where the gas exchanges occur, and then for the systemic vascular bed (Johansen and Burggren, 1980; Tota et al., 1983; Farrell and Jones, 1992). The fish heart represents only 0.04%–0.4% of the total body mass (Johansen and Burggren, 1980; Farrell and Jones, 1992) while the blood volume represents 3%–5% of the total body mass (Olson, 1992).

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The sinus venosus is a thin-walled chamber approximately 60–90 μm thick and similar in volume to the atrium. It receives venous blood through the paired ducts of the Cuvier, hepatic veins, and anterior jugular vein and leads the blood to the atrium. The main component tissue of the sinus wall is the connective tissue, with internal epithelial and external epicardial cover. The amount of muscle fibers varies, and may appear as a thin layer in some species, sparse fibers in most species, or completely absent. Furthermore, the muscle fibers can be cardiac (ex. European eel, *Anguilla anguilla*) or smooth (ex. goldfish, *Carassius auratus*, and common carp, *Cyprinus carpio*), depending on the fish species (Yamauchi, 1980). The main role of the sinus venosus is related to the initiation and control of the heartbeat because it is the site of the specialized pacemaker tissue (sinoatrial node) (Farrell and Jones, 1992).

The atrium is a large, thin-walled trabecular muscle chamber with an irregular shape, capable of generating sufficient pressure only to fill the ventricle, which has a thick muscular wall. Its volume is similar to, or greater than, the ventricular volume and its mass generally constitutes 8%–25% of the ventricle mass and 0.01%–0.03% of the body mass. The atrium is separated from the ventricle by the atrioventricular valve, supported by a ring of cardiac tissue (Farrell and Jones, 1992).

The ventricle is the main heart-generating chamber of pressure and comprises 58%–85% of its weight. It exhibits a large interspecific variability in relation to its mass, morphology, histology, and vascularization, depending on the cardiac demand imposed by the mode of life, level of activity, and the environment (Santer, 1985; Satchell, 1991; Farrell and Jones, 1992). According to Santer (1985), it is possible to distinguish three basic forms of hearts in the different fish species: tubular, saccular, and pyramidal. There is no typical form for the fish's ventricle. In the same way, there is no structural pattern, varying in the different species, from pyramidal to saccular form. In general, it can be said that more sedentary species present a saccular or tubular heart, whereas the more active species often present pyramidal-like hearts (Santer et al., 1983; Tota et al., 1983; Santer, 1985).

The myocardium of pyramidal ventricles always presents two morphological and histologically distinct layers: a spongy endocardium representing the bulk of the ventricular mass, which receives O_2 and nutrients directly from the blood of the ventricular lumen (i.e., venous blood); and an external compact layer supplied with a coronary artery from the arterialized side of the gills. Most fish species have only the spongy layer (Santer and Greer-Walker, 1980).

The fourth chamber, the bulbus arteriosus, is positioned immediately after the ventricle. It consists of elastic connective tissue and collagen and a small amount of smooth muscle cells. It is extremely elastic in order to store and release the kinetic energy generated by the ventricular contraction and to ensure a continuous flow of blood to the secondary lamellae (gas exchange sites) throughout the cardiac cycle. This compliance avoids the transmission of ventricular systolic pressure directly to the secondary lamellae, whose fine unicellular epithelial structures could be ruptured (Johansen, 1962; Stevens et al., 1972; Satchell, 1991; Farrell and Jones, 1992).

The venous blood, coming from the systemic circulation, passes through these chambers and is directed, via the ventral aorta, to the gills where oxygenation occurs (Figs. 9.1–9.3). Fig. 9.4 illustrates the elasticity of the arterial bulb during atrial systole and ventricular diastole and during atrial diastole and ventricular systole.

Tota (1989) proposed the categorization of fish ventricles into four main types based on the existence and arrangement of the spongy and compact layers. The main characteristics distinguishing the four categories of ventricles are the extension

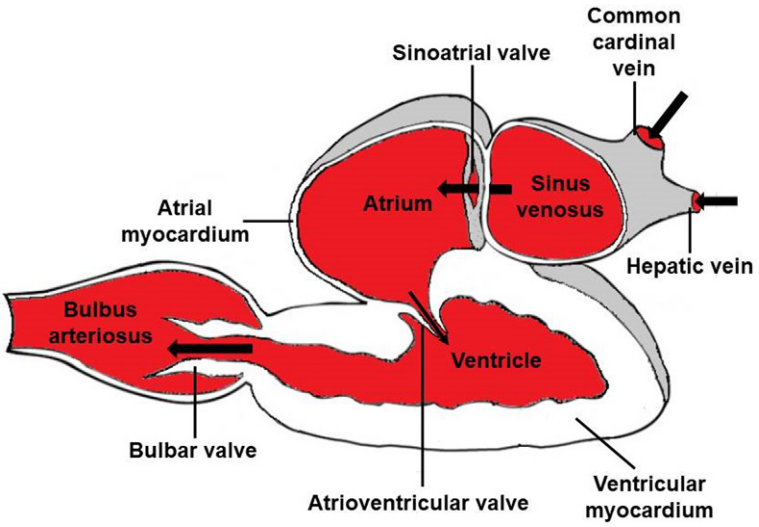

FIG. 9.1 Schematic representation of the longitudinal section of the teleost fish heart, showing the four chambers in series (sinus venosus, atrium, ventricle, bulbus arteriosus). The arrows represent the direction of blood flow through the heart.

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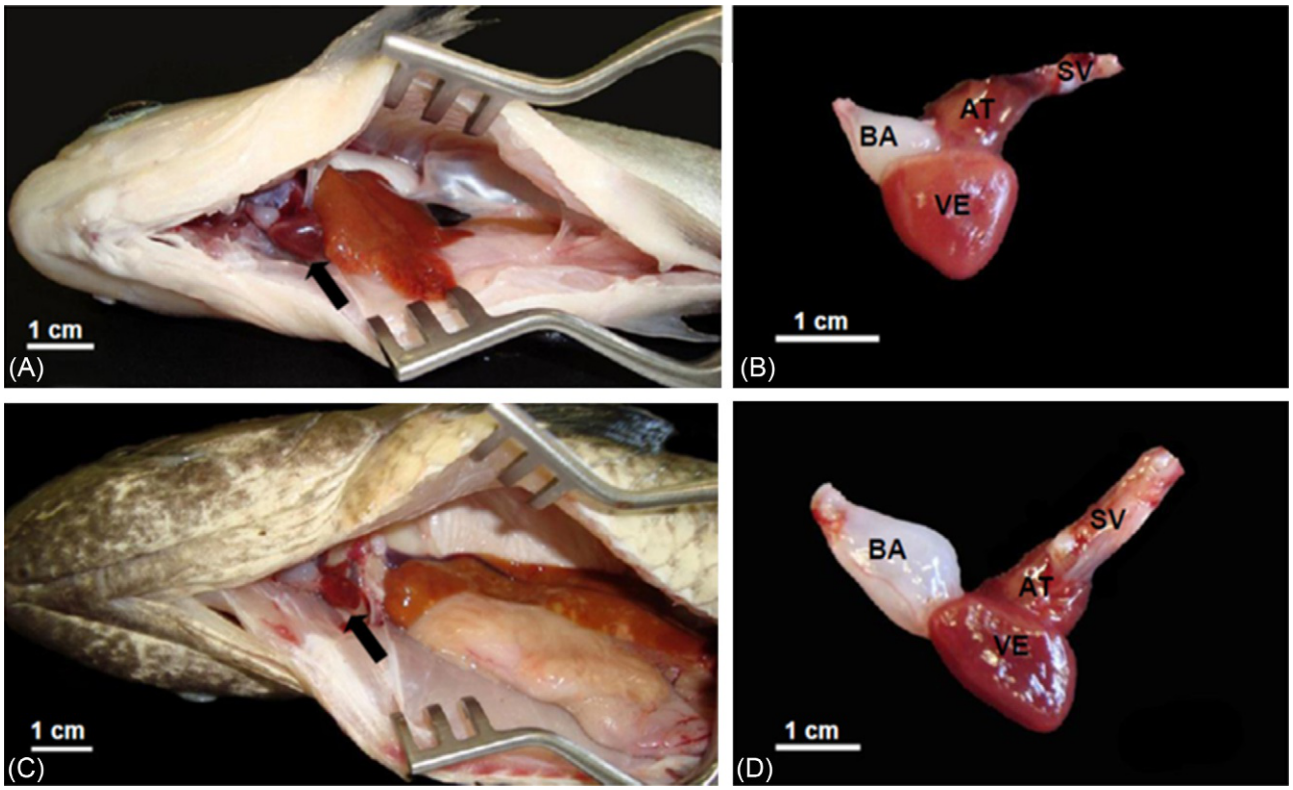

FIG. 9.2 Hearts of matrinxã, *Brycon amazonicus* (A, B), and traíra, *Hoplias malabaricus* (C, D) showing the sinus venosus (SV), atrium (AT), ventricle (VE), and bulbus arteriosus (BA). *(Adapted from Thomaz, J.M., 2012. Habitat, hábito e morfologia cardíaca: influência destes fatores sobre as respostas cardiorrespiratórias à hipóxia e alterações térmicas em espécies de peixes ecologicamente distintas. Tese de Doutorado. Programa de Pós-Graduação em Ciências Fisiológicas, Universidade Federal de São Carlos, São Carlos, SP, Brasil.)*

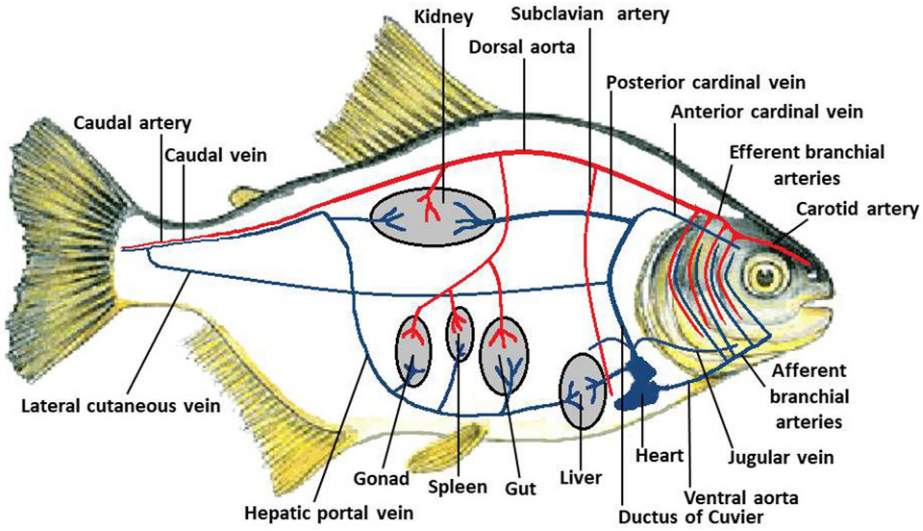

FIG. 9.3 Diagram of the circulatory system of a neotropical fish pacu, *Piaractus mesopotamicus*. Note the branches of the afferent branchial arteries, which carry the venous blood to the gills for gas exchange.

of the spongy to the compact layer and the pattern of vascularization (Fig. 9.5). The type I ventricle has only a spongy myocardium. The three other categories (types II, III, and IV) have compact and spongy myocardial tissue as well as capillaries in the myocardium. Type II is characterized by capillaries of the coronary circulation only in the compact layer. In the ventricles of types III and IV, the coronary circulation reaches both spongy and compact layers. The difference between types III and IV is that type III has less than 30% of compact tissue. The ventricles that have more than 30% of compact and

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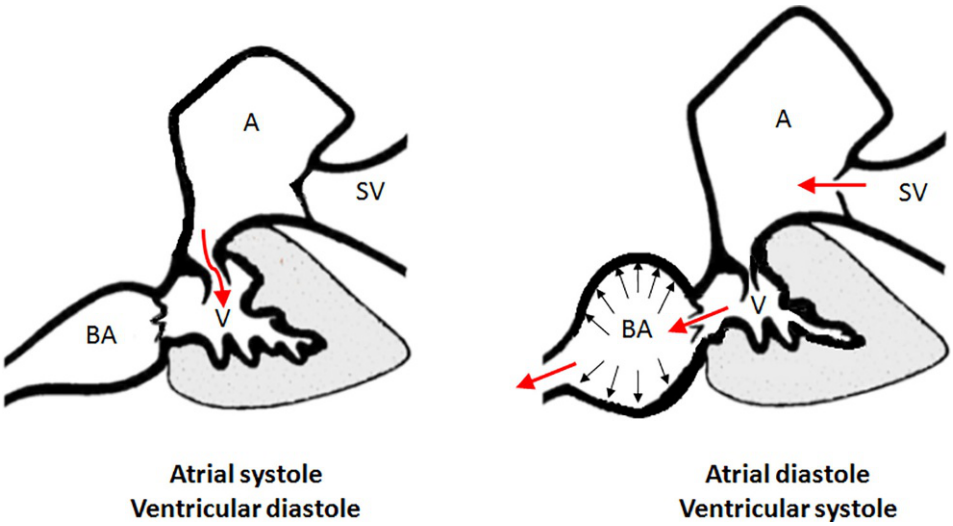

FIG. 9.4 Complacency of the bulbus arterriosus during atrial systole and ventricular diastole and during atrial diastole and ventricular systole. Observe the opening and closing of the valves that separate the different chambers. *SV*, sinus venosus; *A*, atrium; *V*, ventricle; *BA*, bulbus arteriosus. The *arrows* show the blood flow through the cardiac chambers during the different cycles.

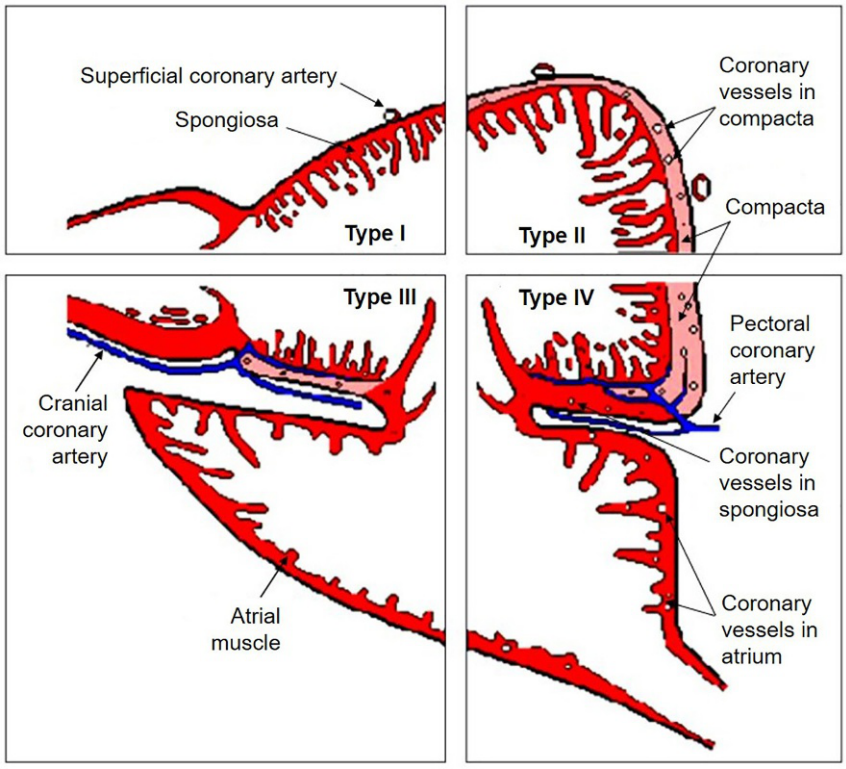

FIG. 9.5 Schematic illustration of the main characteristics distinguishing the four categories of fish ventricles. The basic anatomy of the heart was divided into quadrants, with each one illustrating a particular characteristic of a given type of ventricle. Coronary circulation independent of the ventricle type is derived from the cranial supply, as shown in the lower left quadrant, and an additional pectoral supply in some fish, as shown in the lower right quadrant. *(Source: Farrell, A.P., Jones, D.R., 1992. The heart. In: Hoar, W.S., Randall, D.J., Farrell, A.P. (Eds.), Fish Physiology: The Cardiovascular System, vol. 12A. Academic Press, New York, USA, pp. 1–88.)*

coronaries in the spongy layer are arbitrarily described as type IV. According to Farrell and Jones (1992), most species of fish have only type I ventricles.

The efficiency of the heart's work depends on the sequential activation of the chambers described above. The fish heart, like other vertebrates, is myogenic, possessing a pacemaker tissue that triggers action potentials slowly and spontaneously (Saito, 1973; Haverinen and Vornanen, 2007). Unfortunately, the exact location of the pacemaker area in the fish heart is still not clearly defined. According to Satchell (1991), the location of the pacemaker is not uniform among the different species. In carp, *Cyprinus carpio* (Saito, 1973), and in the Asiatic weatherfish, *Misgurnus anguillicaudatus* (Yamauchi et al., 1973), the primary pacemaker area is located at the junction of the sinus venosus with the atrium, hence the denomination "sinoatrial." Haverinen and Vornanen (2007) only obtained records of potential pacemakers at the base of the

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sinoatrial valve of rainbow trout (*Oncorhynchus mykiss*), where a tissue ring containing myocytes and neural elements was detected by histological methods.

Some characteristics differentiate the fish cardiac muscle cells (myocyte) from those of mammals. Such differences include a small size (1–12.5 μm) compared to mammals (10–25 μm); limited anatomical development of the sarcoplasmic reticulum (SR); peripheral arrangement of myofibrils; and a variable amount of intracellular myoglobin. Among the functional advantages of the occurrence of a small myocyte are a smaller diffusion distance from outside to the center of the cell; and a greater ratio between the surface area of the plasma membrane (sarcolemma) and the intracellular volume, that is, a higher surface/volume ratio. This is of great importance for the exchange of substances, such as calcium, between the intra- and extracellular spaces (Driedzic and Gesser, 1994; Vornanen, 1997).

In addition to the diameter of the myocytes, the transverse tubules (T-tubules), deep sarcolemmal invaginations that facilitate the propagation of the action potential (AP) into the cell (Tibbits et al., 1990) and decrease the diffusional distance of calcium from the extra to the intracellular medium and vice versa (Bers, 2001), also anatomically distinguish mammalian myocytes from other vertebrates. This is due to the fact that fish, like most of the ectotherms studied so far (Keen et al., 1992; Møller-Nielsen and Gesser, 1992; Driedzic and Gesser, 1994), do not present T-tubules. Nevertheless, the higher rates of calcium diffusion, due to the presence of T-tubules in mammals, are partly compensated by the increase in the diffusion rate provided by the smaller diameter of the fish myocytes. According to Bers (2001), from 30% to 50% of the mammalian ventricular sarcolemmal area is concentrated in the T-tubules, where a higher density of calcium channels is also observed.

Another difference of fish myocytes from mammals is the abundance and distribution of the SR, an organelle of the muscle cells specialized in the accumulation of calcium and its release to the contractile apparatus. The SR is relatively well developed in birds and mammals, especially in rats, reflecting the great functional importance of intracellular calcium for myocardial contraction (Chapman, 1983; Bers, 2001; Gwathmey and Morgan, 1991).

The development of SR varies among fish species. In most teleosts (Driedzic and Gesser, 1988; Maylie and Morad, 1995; Chugun et al., 1999) and particularly in the elasmobranchs (Tota, 1989), the SR is generally found distributed in a sparse way. This lower dependence on SR as a supplier of calcium to myofibrils also occurs in other ectothermic vertebrates, such as amphibians and reptiles (Forbes and Sperelakis, 1971). This is also found in the early stages of mammalian ontogenetic development, as seen in neonatal rats (Aho and Vornanen, 1998). SR corresponds to approximately 0.5% of the myocyte volume of fish and amphibians, differing from the approximately 7.3% observed in rats (Helle, 1983; Leknes, 1984).

Furthermore, even in fish species with well-developed SR, such as rainbow trout, this organelle does not display a conspicuous role for Ca^{+2} release in activating contraction at temperatures and heart rates observed in vivo (Hove-Madsen and Gesser, 1989; Hove-Madsen, 1992; Møller-Nielsen and Gesser, 1992; Gesser, 1996; Pelouch and Vornanen, 1996; Shiels and Farrell, 1997; Aho and Vornanen, 1998; Hove-Madsen et al., 1998; Lillywhithe et al., 1999). However, some studies have also indicated that this organelle may contribute to the force generation under physiological conditions (Harwood et al., 2000; Hove-Madsen et al., 2001).

Accordingly, direct SR participation in force generation has also been observed in physiologically relevant cardiac frequencies for several Scombrid fish, such as the tropical tuna *Katsuwonus pelamis* (Keen et al., 1992, 1994; Tibbits et al., 1992; Lillywhithe et al., 1999) and yellow fin, *Thunnus albacares* (Freund and Block, 1998; Shiels et al., 1999), and also in their relatives of temperate environments, such as the Pacific mackerel, *Scomber japonicus* (Shiels and Farrell, 2000) and Atlantic mackerel, *Scomber scombrus* (Santer, 1985). This enables these fish to maintain high heart rates during exercise without significant contractile detriment (Korsmeyer et al., 1997).

Finally, the myofibrils of most fish, although less abundant than in mammals, generally have a peripheral distribution in myocytes (Santer and Cobb, 1972; Vornanen, 1997). Despite the absence of T-tubules, the location of myofibrils in the region closest to the sarcolemma (SL) facilitates the diffusion of calcium from the extracellular space to the contractile apparatus, which is critical for cardiac muscle performance (Farrell and Jones, 1992).

Genesis of cardiac contractility: The pacemaker tissue

The heart of teleost fish, like other vertebrates, is of the myogenic type, that is, the cardiac contractility genesis occurs in a modified and specialized muscle tissue in the heart itself called the pacemaker. As described previously, specialized pacemaker tissue (sinoatrial node—SA) is usually located at the junction of the venous sinus with the atrium. This gives this region crucial importance as the site related to the genesis and control of the heartbeats in the hearts of many species of teleost fish (Farrell and Jones, 1992). However, the pacemaker may be located in other regions or structures. In the hearts of the European eel, *Anguilla vulgaris*, and the African lungfish, *Protopterus ethiopicus*, the pacemaker tissue is located at the

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junction of the venous sinus with the Cuvier duct (Arbel et al., 1977). Differently, in flatfish, *Pleuronectes platessa*, and in the brown bullhead, *Ameiurus nebulosus*, this tissue is located at the atrioventricular junction (Laurent, 1962; Santer, 1972; Cobb and Santer, 1973). As in the hearts of other vertebrates, in fish, even in the absence of a specialized pacemaker tissue, the myogenic activity of the atrium, ventricle, or atrioventricular region can also initiate contraction of the cardiac fibers, but at a lower frequency and a more irregular rate than the pacemaker tissue (Farrell and Jones, 1992).

The SA node, in general, consists of a cluster of modified myocytes located in the sinoatrial canal, close to atrial myocytes and with few striations. This tissue is richly innervated by postganglionic vagal fibers whose endings have vesicles containing acetylcholine (Satchell, 1991). Their cells are characterized by an unstable resting potential. During the slow diastolic depolarization, or prepotential, there is a discrete and constant influx of Na^+ through the sarcolemma until it reaches a discharge level, when the action potential occurs. The membrane potentials of pacemaker cells can be recorded by means of intracellular microelectrodes and result in tracings, as shown in Fig. 9.6.

The APs of the SA node are divided into three phases. Phase 4 is the spontaneous depolarization (pacemaker potential) that triggers the AP as soon as the membrane potential reaches the threshold. At the beginning of the depolarization when the membrane potential is very negative (around −60 mV), the ion channels are opened, resulting in a slow influx of Na^+. This influx causes a gradual and spontaneous increase in depolarization until it reaches the threshold, initiating phase 0. Simultaneously, when the membrane potential reaches values around −50 mV, another type of channel opens. This Ca^{2+} channel is known as transient or type T-channel. As Ca^{2+} enters the cell through these channels, its electrochemical gradient decreases and the Ca^{2+} inflow current will further depolarize the cell. As the membrane continues to depolarize to about −40 mV, more Ca^{2+} channels open. The opening of these channels causes more Ca^{2+} influx into the cell, which continues to depolarize until the action potential threshold is reached (usually between −40 and −30 mV). During Phase 4, there is also a slow decline in K^+ output as the K^+ channels, responsible for Phase 3, continue to close. This decrease in K^+ conductance contributes to the pacemaker depolarizing potential (Nakayama et al., 1984; Matsuura et al., 2002; Klabunde, 2005).

Phase 0 of the depolarization is caused primarily by an increase of Ca^{2+} conductance through the L-type channels, which begin to open at the end of Phase 4. Because the Ca^{2+} entry through these channels occurs more slowly, the rate of depolarization is much slower than that found in other cardiac cells. Phase 3 is initiated immediately after the AP peak.

During phase 3, the membrane repolarization occurs as the K^+ channels open and the K^+ conductance increases, thus increasing the hyperpolarizing K^+ currents directed toward the outside of the cell. Simultaneously, the L-type Ca^{2+} channels are inactivated and close, decreasing both the Ca^{2+} conductance and the depolarization currents. During depolarization, the membrane potential approaches the equilibrium potential for Ca^{2+}, which generally reaches a value above +100 mV. During repolarization, the Ca^{2+} conductance decreases and K^+ increases, which brings the membrane potential near the equilibrium potential to K^+, which is generally just above −100 mV. Therefore, the action potential in SA cells is essentially dependent on changes in the conductance to Ca^{2+} and K^+ (Ju and Allen, 1998; Klabunde, 2005; Cordula et al., 2006). When the pacemaker cell is fully repolarized, the cycle starts spontaneously. The heart rate, therefore, is determined by the pacemaker activity rate, that is, by the number of action potentials generated per unit of time. In general, the heart rate is expressed in beats per minute (bpm).

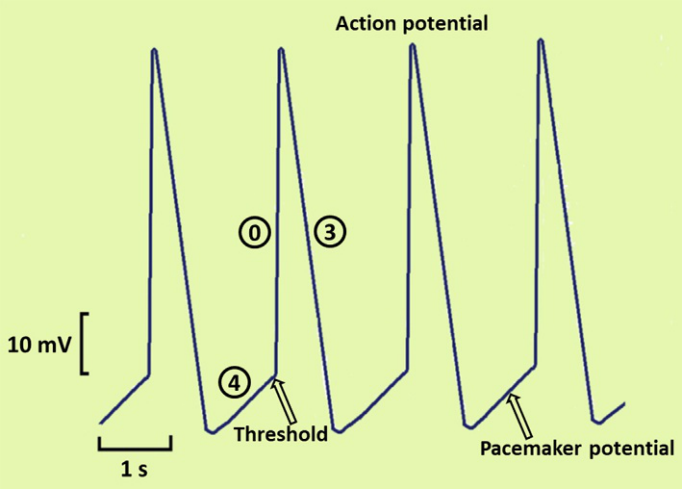

FIG. 9.6 Diagram of action potentials (AP) of pacemaker cells of fish sinoatrial tissue showing the phases of electrical events, the prepotential with spontaneous depolarization, and the threshold above which depolarization of the AP occurs.

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Although the pacemaker activity is spontaneously generated by SA node cells, the rate of this activity can be significantly modified by external factors, such as adrenaline, acetylcholine, drugs, ion concentrations, temperature, and hypoxia. The effects of these last two factors on the heart of teleost fish will be detailed below.

The action potential of atrial and ventricular myocytes

Atrial and ventricular myocytes are "nonpacemaker" cells and have characteristic action potential. Because these cells depolarize very fast, their action potentials are known as "fast response action potential." Unlike the pacemaker cells, myocytes have a stable membrane resting potential (Phase 4—Fig. 9.7). The membrane resting potential is quite negative during Phase 4 (in the trace example of Fig. 9.8, around −70 mV). This occurs because the K^+ channels are opened, generating high conductance and K^+ current. Phase 4 is associated with K^+ currents as Na^+ are exiting the myocyte, leaving the membrane potential more negative internally. Concomitantly, the fast sodium channels and the slow calcium channels (L-type channels) are closed. The depolarization phase of myocytes is very fast (Phase 0—Fig. 9.7). This fast depolarization is

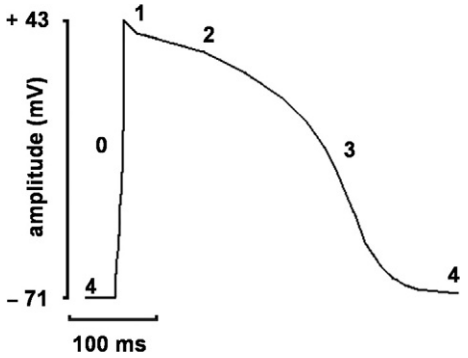

FIG. 9.7 Action potential recorded in the isolated myocyte of rainbow trout stimulated at 0.4 Hz, showing the different phases of depolarization and repolarization. *(Adapted from Patrick, S.M., White, E., Shiels, H.A., 2010. Mechano-electric feedback in the fish heart. PLoS One 5(5), e10548).*

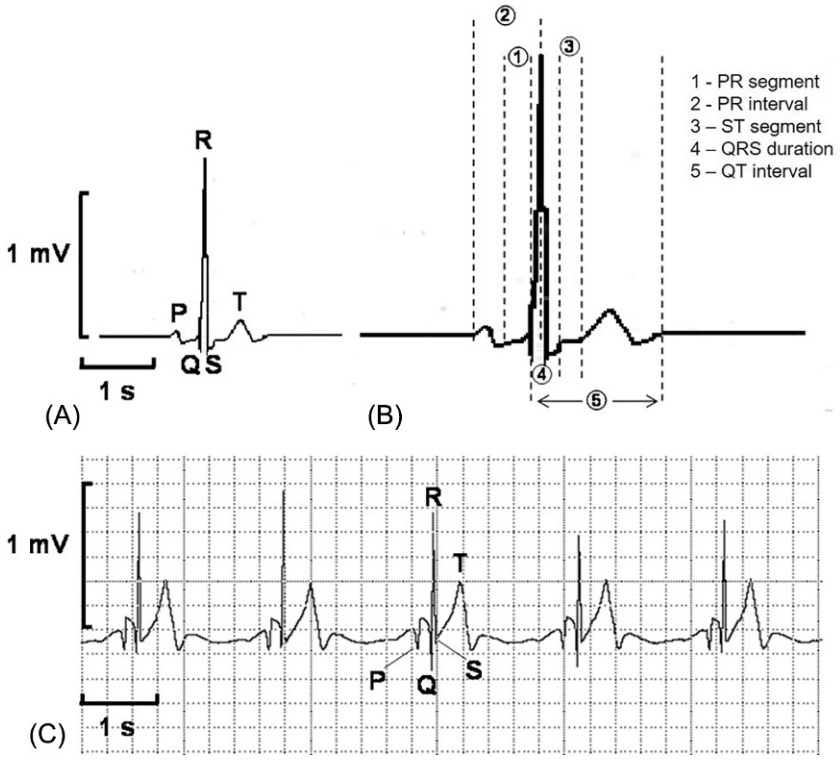

FIG. 9.8 (A) Electrocardiogram (ECG) of pacu, *Piaractus mesopotamicus*, recorded in the DI lead of the standard electrocardiography, showing P wave (corresponding to depolarization of atrial fibers), QRS complex (corresponding to depolarization of ventricle fibers), and T wave corresponding to the repolarization of the ventricle fibers). (B) Pacu electrocardiogram showing the duration of the waves, segments, and intervals used in the analysis of cardiac function. (C) Electrocardiogram of African catfish, *Clarias gariepinus*, recorded in the DI lead, at 25°C and under normoxic conditions.

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due to a transient increase in Na^+ conductance through the fast sodium channels. At the same time that the Na^+ channels open, the K^+ channels close, decreasing both conductance and the K^+ current. Phase 1 (Fig. 9.7) represents the onset of repolarization caused by the opening of the K^+ channels, which induces K^+ to exit, causing a brief and fast membrane repolarization. However, due to a simultaneous increase in Ca^{2+} entry, the repolarization is delayed and enters into a plateau phase (Phase 2). This prolonged phase of repolarization differentiates the AP of the cardiac fiber from the other action potentials that occur in the membranes of skeletal muscles and neurons. This plateau allows the atrium and the ventricle to contract at different times. Phase 3 is characterized by the closure of the L-type Ca^{2+} channels and the opening of the K^+ channels. This causes a rapid repolarization, bringing the membrane potential back to its resting condition, or Phase 4 (Klabunde, 2005).

Once the AP is started, there is a time period comprising Phases 1 and 2 and part of Phase 3 in which no other AP can be initiated. This period of time is called the absolute refractory period and functions as a protective mechanism of the heart, preventing the occurrence of multiple APs and, consequently, exacerbated increases in heart rate and even the tetanization of the heart muscle.

The action potential conduction system

In mammals, the conduction system of the AP generated in the pacemaker tissue to the atrial and ventricular fibers is well known and histologically well identified. This system involves the bundles of His and the Purkinje fibers, whose cells differ histologically from atrial and ventricular myocytes. This differentiated tissue has not yet been identified histologically in fish. However, there is physiological evidence suggesting that the conduction of AP through myocytes occurs in a coordinated way as well as in the hearts of other vertebrates. Because there is no pattern of AP conduction in the fish heart, the evidence indicates that this phenomenon is species-specific. In tench, *Tinca tinca*, for example, the APs first appear on the dorsal surface of the ventricle around the atrioventricular junction (AV junction), and propagate rapidly to the left side and then to the ventral surface and downward toward the apical region. From this region, the APs propagate more slowly through the other portions of the ventricle to the bulbar region, where they have initiated. This process takes approximately 80 ms (Ripplinger and Pierron, 1973). In rainbow trout and the European eel, the left side of the ventricle and the apex are activated earlier than the right side (Chiesa et al., 1962); pacu, *Piaractus mesopotamicus*, traíra, *Hoplias malabaricus*, and trairão, *Hoplias lacerdae*, seem to follow this same pattern (Rantin et al., 1995).

An important aspect of the conduction process is the so-called atrioventricular delay, or A-V delay. In higher vertebrates, the atria are separated from the ventricles by fibrous rings. These rings hinder the passage of electrical activity from the atria to the ventricles. This delay is important so that the atria are emptied to the maximum (atrial systole) and the ventricles, in diastole, are completely filled before systole starts. In other words, the A-V delay is important to ensure that the atrial and ventricular systoles occur at different times, ensuring the role of the heart as a pump. There are few reports in the literature about the composition of the tissue separating the atrium from the ventricle in fish. In the flatfish, *P. platessa*, for example, the A-V delay is probably caused by a fibrous portion and bands of smaller diameter myocytes that separate their chambers (Santer and Cobb, 1972). However, it is unclear whether this tissue arrangement is common to all teleosts. Although the details of the A-V delay in fish are not fully known, electrocardiographic studies to date indicate that it occurs efficiently in practically all species.

Electrocardiogram

Due to the good conductivity of body fluids and tissues, the electrical phenomena occurring in myocardial fibers during the cardiac cycle can be recorded on the body surface using electrocardiography. The teleost fish present electrocardiographic waves with morphology typical of the other vertebrates: a P wave (corresponding to atrial depolarization), a QRS complex (corresponding to ventricular depolarization), and a T wave (corresponding to ventricular repolarization—Fig. 9.8A). The analysis of the morphology and duration of waves and their respective segments and intervals (Fig. 9.8B) can provide important information on the cardiac function of the fish.

The excitation-contraction coupling

The sequence of events occurring from the electric excitation of the surface membrane (AP) in the sarcolemma of the muscle cell to contraction of the heart is called excitation-contraction coupling (E-C coupling). The AP of the muscle cell is initiated with the opening of the Na^+ channels of a certain myocyte, due to the local electrical change, and consequent passage of Na^+ ions through the communicating junctions (GAP junctions) to adjacent myocytes. The local positivation

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changes the electrical potential of ions near the communicating junction, which results in the opening of voltage-dependent Na^+ and Ca^{2+} channels in the sarcolemma (SL). These channels have an intracellular domain with positive amino acids, which can promote changes in their conformation with the modification of the electric potential (Bers, 2000). The influx of Ca^{2+} in the myocytes is mainly due to L-type (slow) channels. These channels open and close more slowly than the Na^+ channels, which results in the maintenance of a prolonged positive membrane potential characteristic of cardiac AP (Bers, 2000).

Another important component of the cardiac E-C coupling is the Na^+/Ca^{2+} (NCX) exchanger, a reversible countertransport system with stoichiometry from 3 Na^+ to 1 Ca^{2+} ions. The direction and amplitude of transport by NCX depend on the membrane potential as well as the intra- and extracellular concentrations of Na^+ and Ca^{2+}: high concentrations of cytosolic Ca^{2+} facilitate its efflux (NCX operating in normal mode—Na^+ influx) while positive membrane potentials and high intracellular Na^+ concentrations enable the influx of Ca^{2+} (NCX operating in reverse mode—Na^+ efflux) (Bers, 2002).

Anatomic features such as the small diameter of the myocytes associated with the absence of T-tubules and the limited anatomical development of SR result in peculiarities in the EC coupling in the myocardium of ectothermic vertebrates, particularly in relation to the origin of Ca^{2+} to be used by the contractile apparatus (for a review, see Tibbits et al., 1992 and Vornanen et al., 2002).

A general scheme of cardiac E-C coupling is shown in Fig. 9.9, which compares the model developed for mammals to that known so far for fish, based on that described for teleosts. The main difference between ectotherms and mammals is related to the origin of calcium reaching the myofibrils. It has been known for more than a century (Ringer 1883 *apud* Bers, 2001) that extracellular calcium is essential for myocardial contraction. The Ca^{2+} influx by SL, both through NCX and dihydropyridine-sensitive voltage-dependent L-channels (DHPR), plays a critical role in the regulation of contractility (Bers et al., 1988; Mikami et al., 1989; Romanin et al., 1992; Nakai et al., 1997).

According to Fabiato (1983), this transarcolemmal Ca^{2+} influx triggers, in mammals, the release of an even larger amount of this ion from SR, through Ca^{2+} channels called ryanodine channels (RyR). This mechanism is called calcium-induced calcium release (CICR) (Bers, 1987). Moreover, SR Ca^{2+} efflux may also occur in the myocardium by elevated intracellular levels of inositol (1,4,5) triphosphate (IP3) caused by the binding of an agonist, such as catecholamines, to SL (Berridge, 1993).

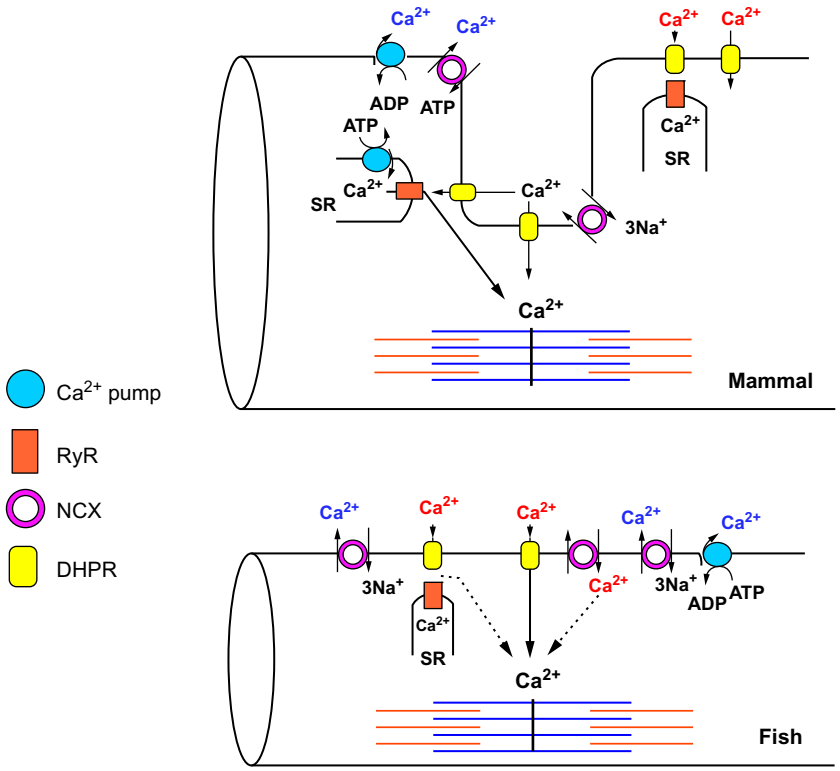

FIG. 9.9 General diagram of the E-C coupling mechanism of the cardiac muscle, comparing the existing model for mammals with that proposed for teleosteal fish. The figure also illustrates some differences between the structure of fish and mammalian myocytes, such as variations in cell diameter, distribution, and abundance of SR and the existence of T-tubules. The arrows indicate the direction of Ca^{2+} flow. Dotted lines suggest a possible role for calcium sources. *DHPR*, dihydropyridine channel; *SL*, sarcolemma; *SR*, sarcoplasmic reticulum; *NCX*, Na^+/Ca^{2+} exchanger. *(Modified from Tibbits, G.F., Moyes, C.D., Hove-Madsen, L., 1992. Excitation-contraction coupling in the teleost heart. In Hoar, WS., Randall, D.J., Farrell, A.P. (Eds.). Fish Physiology, vol. XIIA. Academic Press, New York, USA, pp. 267–304).*

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In addition to the interspecific variability in the magnitude of Ca^{2+} influx through the L channels of the SL in mammals (Bers, 1985), most studies indicate that this transarcolemmal calcium entry is insufficient to allow contraction (Chapman, 1983; Driedzic and Gesser, 1994; Bassani et al., 1994b, 1996). Thus, the Ca^{2+} liberated from SR is the main source of this ion for the contraction in the heart of all mammals studied so far (Bers, 1985; Wier, 1990; Gwathmey and Morgan, 1991; McCall and Orchard, 1991; Bassani et al., 1992, 1993, 1994a, b; Bers et al., 1993; Klautz et al., 1997).

This situation seems to be very different in the hearts of ectothermic vertebrates. Tibbits et al. (1991) found that in amphibian myocardium, Ca^{2+} released from SR is not sufficient to promote contraction. Besides, the occurrence of CICR in the ventricles of these animals has been contested by several authors (Fabiato and Fabiato, 1978; Fabiato, 1983). Ventricular contraction in both amphibians (Morad and Orkand, 1971; Bers, 1985; Gwathmey and Morgan, 1991; Mill et al., 1992) and in most fish (Driedzic and Gesser, 1988; Hove-Madsen and Gesser, 1989; Møller-Nielsen and Gesser, 1992; Keen et al., 1994; Gesser, 1996; Rantin et al., 1998; Costa et al., 2004) appears to be relatively insensitive to the alkaloid ryanodine (inhibitor of SR function), even when used in concentrations that are effectively blocking the release of Ca^{2+} from SR (Sutko and Airey, 1996) in experiments using both stimulation frequencies and physiological temperatures for each animal.

Due to such characteristics and the higher surface/volume ratio, which also provides a higher density of DHPR receptors (Thomas et al., 1996), it is expected that in the heart of ectothermic vertebrates, including fish, the transarcolemmal transport of Ca^{2+} is sufficient to allow contraction (Forbes and Sperelakis, 1971; Shepherd and Kavaler, 1986; Vornanen, 1997), unless another organelle, such as mitochondria, also contributes to the regulation of the beat-to-beat of the intracellular concentration of Ca^{2+}. However, it is noteworthy that a possible direct participation of the mitochondria in the E-C coupling has been ruled out for the mammalian heart based on a variety of kinetic and pharmacological arguments (Fabiato, 1983; Driedzic and Gesser, 1994; Bassani et al., 1996). However, the validity of these arguments still needs to be verified in fish.

Therefore, the role of SR in the generation of tension by the hearts of most ectothermic vertebrates, including the species of temperate fish already studied, is usually disregarded (Tibbits et al., 1990; Møller-Nielsen and Gesser, 1992; Hove-Madsen et al., 1998). However, SR participation in the management of tropical fish Ca^{2+} has been shown to be more variable, showing no correlation between the activity level of the animal and the SR functionality, as described for temperate species. This is exemplified by the results obtained for Neotropical teleost species such as traíra (Olle, 2003; Rivaroli et al., 2006), pacu, (Anelli-Jr et al., 2004), curimbatá, *Prochilodus lineatus* (Rivaroli et al., 2006), and the marbled swamp eel, *Synbranchus marmoratus* (Rocha et al., 2007a, b), whose SRs are functional, despite the different levels of activity of these species. In this way, the hypothesis that there is a phylogenetic component determining the SR functionality in fish cannot be rejected. In ecologically distinct tropical species, with different habits and occupying different habitats, such as matrinxã (*Brycon amazonicus*—active species of lotic environments) and traíra (sedentary species of lentic environments), the SR contributes with about 80% and 70% of the Ca^{2+} activator for the development of force during the excitation-contraction coupling (Monteiro et al., 2017). The graphical representation of the relative contribution of Ca^{2+} accumulated in SR to the generation of contraction force as a function of the stimulation frequency is presented in Fig. 9.10 in preparations with and without ryanodine.

In both mammals and fish, cardiac relaxation occurs by reducing the cytosolic Ca^{2+} concentration to the diastolic level of about 100 nM (Tibbits et al., 1992). The decrease of free Ca^{2+} occurs through the activity of transport systems located in three different cellular compartments: (1) Ca^{2+}-ATPase from SR (SERCA); (2) Na^{+}/Ca^{2+} exchanger (NCX) and Ca^{2+} -ATPase of sarcolemma; and (3) mitochondria (Hasenfuss et al., 1996; Misquitta et al., 1999).

In the hearts of mammals, SERCA and NCX are the main mechanisms to reduce the cytosolic concentration of Ca^{2+} in order to promote maximum relaxation. According to Bers (2001), under normal conditions, about 70% of the cytosolic Ca^{2+} is removed by SR through SERCA and the remainder by NCX, which is the main efflux medium of this cell ion. Moreover, SL Ca^{2+}-ATPase and the mitochondrial Ca^{2+} outlet seem to play a very small role in mammalian cardiac relaxation (Bassani et al., 1996; Marengo et al., 1997).

In ectothermic vertebrates, the Ca^{2+} entering the cell from the transarcolemmal flow is removed from the cytosol during cardiac relaxation, mainly through sarcolemmal NCX and Ca^{2+}-ATPase (Tibbits et al., 1991; Thomas et al., 1996; Xue et al., 1999), the first constituting the main mechanism in teleosts (Driedzic and Gesser, 1994). Therefore, during the cardiac cycle of fish, we can observe a reduced importance of SR both as a supplier and as a store of calcium. The opposite occurs with SL. However, this premise is based primarily on the literature on temperate fish, making it essential to obtain detailed knowledge of the cardiac E-C coupling of neotropical and tropical fish. As previously mentioned, studies with some of these species point to a significant role of SR as a Ca^{2+} supplier for the development of myocardial contraction force.

As previously described, the activation of myofilaments during EC coupling occurs in response to an increase in cytosolic Ca^{2+} concentration, which may depend on both the mobilization of intracellular Ca^{2+} from the SR and the influx of Ca^{2+} by SL through the Ca^{2+} voltage-dependent channels (L channels) and NCX (Hove-Madsen et al., 2000). On the

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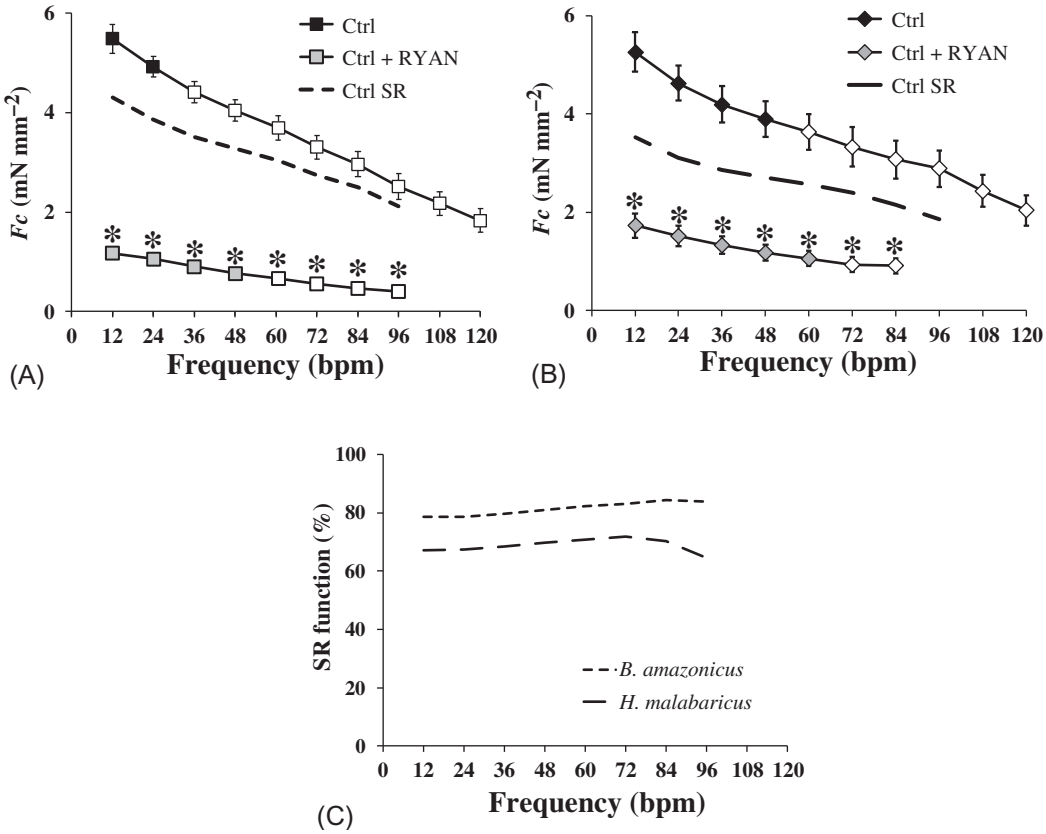

FIG. 9.10 Isometric force (Fc) developed by ventricular strips from specimens of (A) matrinxã, *Brycon amazonicus*, and (B) trahira, *Hoplias malabaricus*, at different stimulation frequencies in the absence (Ctrl) or presence of 10 μM ryanodine (Crtl+RYAN). Mean values ± SEM. Open symbols denote a significant difference in relation to the values obtained at 12 bpm while asterisks indicate significant differences between experimental groups at the same frequency. The role of sarcoplasmic reticulum (SR) as a proportion of Fc generation is shown in (C). *(Source: Monteiro, D.A., Taylor, E.W., Rantin, F.T., Kalinin, A.L., 2017. Impact of waterborne and trophic mercury exposures on cardiac function of two ecologically distinct Neotropical freshwater fish Brycon amazonicus and Hoplias malabaricus. Comp. Biochem. Physiol. C 201, 26–34).*

other hand, the cardiac muscle relaxes by reducing the cytosolic levels of Ca^{2+} to resting diastolic levels, mainly by its transport to the extracellular space through SL (especially via NCX) or to SR by SERCA activity.

The time required for both the increase and the reduction of the cytosolic Ca^{2+} concentration as well as the amount of this ion that is cyclized beat-to-beat depends directly on the degree of activity of the Ca^{2+} transport mechanisms in each compartment, which varies according to species and temperature (Gwathmey and Morgan, 1991; Vornanen, 1998).

Heart rate, systolic volume, and cardiac output

The function of the heart is to supply blood to the tissues and to generate and sustain the systolic pressure necessary for adequate tissue perfusion. The heart contraction by its muscular walls must generate sufficient pressure to propel blood from the cardiac chamber (ventricle) to the ventral aorta. Besides, these contractions must occur with a certain frequency so that the blood constantly reaches the tissues. The number of cardiac contractions per unit of time required to maintain adequate blood supply to tissues is called the heart rate (f_H). The fH is generally expressed in beats per minute (bpm). For each beat, a certain volume of blood is ejected into the bulbus arteriosus and then into the ventral aorta. This volume is called systolic volume (*SV*) and is usually expressed in mL of blood per kg of animal weight (mL kg^{-1}). *SV* multiplied by f_H results in cardiac output (*Q*), or minute volume that corresponds to the volume of blood released by the ventricle to perfuse the tissues every minute (mL min^{-1} kg^{-1}). Thus, any change in both f_H and *SV* will influence the other cardiac variables. In addition, the *Q* is determined not only by the heart activity, but also by its peripheral resistance (Klabunde, 2005). Although cardiac function is well known and established for mammals, in fish this function presents a number of controversies. These disagreements result from studies whose methods are not well standardized, having been conducted at different temperatures, and also by the great

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interspecific variability. The values of Q, f_H, and SV of different species of teleosts obtained in different temperature conditions and through different techniques of cardiac function evaluation are presented in Table 9.1.

Control of cardiac function

Although the heart is endowed with its own rhythmicity, f_H, VS, and Q need to be adjusted to distribute blood appropriately to different tissues to maintain specific metabolic demands. Therefore, the fish heart is generally innervated by fibers of both the sympathetic and parasympathetic autonomic nervous system. The innervation of the teleost fish heart is similar to that of mammals, with inhibitory vagal cholinergic innervation acting via muscarinic receptors (Smith et al., 1985; Axelsson and Nilsson, 1986; Axelsson et al., 1987) and excitatory autonomic adrenergic spinal innervation, acting via β-adrenergic receptors (Holmgren, 1977; Cameron, 1979). However, this kind of innervation cannot be generalized to teleosts. As an example, the hearts of the sole (Pleuronectidae) do not seem to have adrenergic innervation (Donald and Campbell, 1982). This suggests that not all teleosts have an adrenergic nervous supply.

Fig. 9.11 shows the effects of adrenaline and acetylcholine on the action potential of pacemaker cells. These mediators act antagonistically on the permeability of the membrane to the influx of Na^+ during Phase 4 of the action potential. Thus, adrenaline increases the permeability of the membrane to this ion, causing a shift in the slope of that phase to the left. As a consequence, a greater number of action potentials per unit of time occurs, which, transmitted to the entire atrial and ventricular myocardium, causes an increase in f_H. In contrast, acetylcholine decreases the permeability of the membrane to Na^+, displacing the slope to the right, consequently causing a decrease in f_H.

Effect of temperature

Because fish are ectothermic animals and because of the high conductivity and high specific heat of the water, most fish species have a body temperature practically identical to the environment. Some species live at almost constant temperature in deep marine waters and in the polar oceans. In these species, temperature has almost no influence on cardiac function. Other species, however, are constantly exposed to changes in ambient temperature, which causes major changes in their physiological, biochemical, metabolic, and behavioral processes. Depending on the adaptation to temperature, lifestyle, and activity level, teleosts may exhibit large changes in cardiac function. Seasonal or acute thermal fluctuations, such as vertical movement in the water column through the thermocline, can cause a significant and rapid oscillation of body temperature, substantially influencing the cardiovascular function of the fish.

The temperature affects the permeability of the membranes of the pacemaker cells. While high temperatures induce an increase in membrane permeability, cold causes a decrease in permeability. Consequently, the heat shifts the slope of Phase 4 (see Figs. 9.6 and 9.11) to the left, causing an increase in heart rate. Conversely, cold shifts the slope to the right, causing a decrease in heart rate (see the review by Tibbits et al., 1992).

Like the pacemaker tissue cells, the temperature also affects the atrial and ventricular myocytes. As temperature acts on myocyte metabolism and, more directly, on the permeability of their membranes, the duration of action potentials is inversely proportional to temperature, that is, low temperatures cause an increase in AP duration as a whole whereas heat acts in the reverse way (Fig. 9.12). An increase in the AP duration particularly affects the duration of Phase 2 (plateau), consequently increasing the absolute refractory period (Lennard and Huddart, 1991; Aguiar et al., 2002).

For most teleost species, the f_H is limited within a range of 10–60 bpm and generally does not exceed 120 bpm (Farrell, 1991; Anelli-Jr et al., 2004). However, much higher f_H values were described by Brill (1987) for scombrid species such as *T. albacares* (90 to 160 bpm), *Euthynnus affinis* (175 to 253 bpm), and *K. pelamis* (191 to 237 bpm). The highest f_H occurs when these fish reach the highest swimming performance. Another example is the gobid fish, *Bathygobius soporator*, which inhabits the intertidal regions of the Brazilian littoral. During low tide, when trapped in sea pools on days of great sunshine, it can experience temperatures of 40°C or more. When exposed to acute temperature transitions of 25–35°C, this species progressively and significantly increases its f_H, reaching values of approximately 225 bpm (Rantin et al., 1998). The effect of temperature on the f_H of several tropical species is shown in Fig. 9.13.

The temperature coefficient Q_{10} provides important information about the rate of change that occurs in a chemical or biological system when there is an increase of 10°C in temperature. This coefficient is calculated through the so-called Van't Hoff equation for Q_{10}:

$$Q_{10} = (R_2/R_1)^{10/(T2-T1)}$$

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TABLE 9.1 Values of cardiac output (Q), heart rate (f_H), and systolic volume (SV) of different species of teleosts, at different temperatures and obtained with different techniques for evaluating cardiac function

Espécie	Temp (°C)	Weight (g)	Q (mL kg^{-1} min^{-1})	f_H (bpm)	VS (mL kg^{-1})	Method	Reference
Cpmmon carp *Cyprinus carpio*	6	~1110	9.5	7	1.22	In vivo—rest	Stecyk and Farrell (2002)
	10	~1230	19	12	1.58	In vivo—rest	
	15	~790	15	17	0.77	In vivo—rest	
"European seabass *Dicentrarchus labrax*	18	250–320	91	65–70	1.38	In situ—perfused	Farrell et al. (2007)
	22	250–320	95	95–100	1.05	In situ—perfused	
Atlantic cod *Gadus morhua*	5	~1910	9	16	0,52	In vivo—rest	Webber et al. (1998)
	5	~1910	31	28	1.12	In vivo—swimming	
	10	~1910	12	28	0,45	In vivo—rest	
	10	~1910	35	40	0.88	In vivo—swimming	
	10	861–1335	22	36	0.60	In vivo—swimming	Gollock et al. (2006)
	20	861–1335	47	73	0.67	In vivo—rest	Gollock et al. (2006)
Skipjack tuna *Katsuwonus pelamis*	25	1600	138[a]	125.9	1.1	Sectioned	Bushnell and Brill (1992)
	25	1300	105[a]	154	0,68	In vitro—perfused	Farrell et al. (1992)
Rainbow trout *Oncorhynchus mykiss*	4	~1120	5	38	0.14	In vivo—rest	Taylor et al. (1996)
	4	~1120	14	44	0.25	In vivo—swimming	
	11	~822	21	52	0.53	In vivo—rest	
	11	~822	73	59	1.35	In vivo—swimming	
	18	~810	20	91	0.25	In vivo—rest	
	18	~810	41	95	0.50	In vivo—swimming	
	10	~500	15	55	0.27	In situ—perfused	Overgaard et al. (2004)
	10	290–1060	100[b]	47	100[b]	In vivo—rest	Sandblom and Axelsson (2007)
	13	290–1060	120[b]	62	91[b]	In vivo—rest	
	16	290–1060	131[b]	76	80[b]	In vivo—rest	
Flatfish *Pleuronectes americanus*	4	453–843	9.8	21	0.5	In vivo—rest	Joaquim et al. (2004)
	4	453–843	25.4	31.6	0.80	In vivo—swimming	
	10	453–843	15.5	34	0.5	In vivo—rest	
	10	453–843	39.2	52.4	0.74	In vivo—swimming	
Albacore *Thunnus alalunga*	16.5	9100	28[a]	87	0.32	Anesthetized	Lai et al. (1987)
	21.9	9300	44[a]	115	0.38	Anesthetized	White et al. (1988)
Yellowfin tuna *Thunnus albacares*	25	1400	126[a]	96.7	1.3	Sectioned	Bushnell and Brill (1992)
	25	1400	75[a]	112	0.67	Anesthetized	Jones et al. (1993)
Pacific bluefin tuna *Thunnus orientalis*	5	~6700	19	23	0.86	In situ—perfused	Blank et al. (2004)
	10	~6700	21	36	0.57	In situ—perfused	
	15	~6700	20	55	0.37	In situ—perfused	
	20	~6700	17	78	0.23	In situ—perfused	
	25	~6700	18	100	0.18	In situ—perfused	

[a]*Data calculated based on the values of* f_H *and* VS.
[b]*Data expressed as % of initial value at 10°C.*
Adapted from Kalinin, A.L., Costa, M.J., Rantin, F.T., Glass, M.L., 2009. Effects of temperature on cardiac function in teleost fish. In M.L. Glass, and S.C. Wood, (Eds.). Cardio-Respiratory Control in Vertebrates. Springer-Verlag, Berlin, Germany, pp. 121–160.

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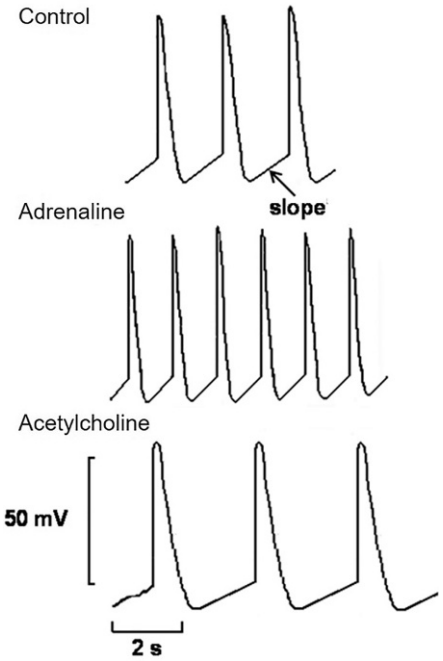

FIG. 9.11 Effect of adrenaline and acetylcholine on the frequency of action potentials of the teleostome heart pacemaker cells. Observe the shift of the phase 4 slope to the left (adrenaline effect) and to the right (acetylcholine effect).

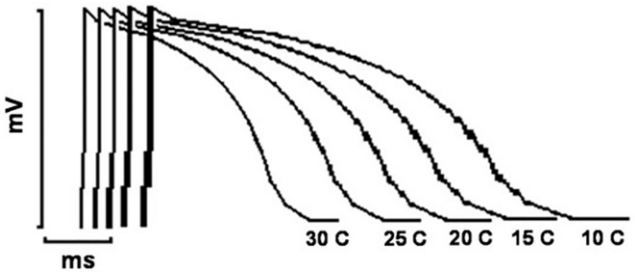

FIG. 9.12 Representation of the effect of temperature on the atrial and ventricular myocyte action potentials of fish.

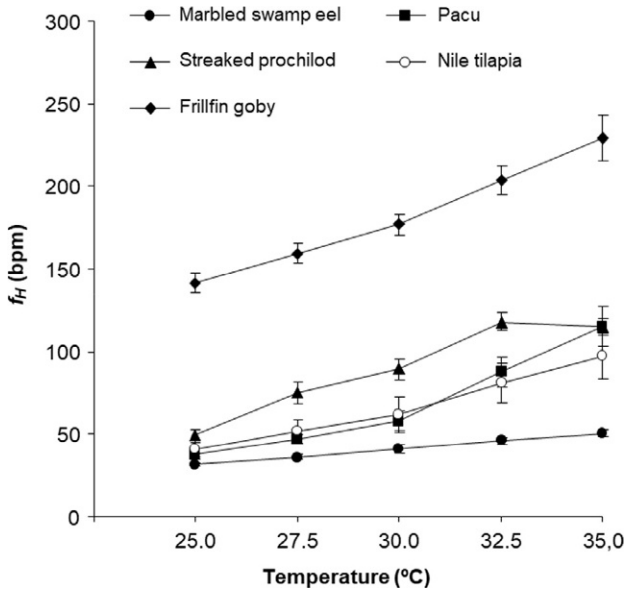

FIG. 9.13 Effect of temperature on heart rate (f_H) of frillfin goby, *Bathygobius soporator* (Rantin et al., 1998), curimbatá, *Prochilodus lineatus* (Kalinin et al., 2009), Nile tilapia, *Oreochromis niloticus* (Costa et al., 2000), pacu, *Piaractus mesopotamicus* (Anelli-Jr et al., 2004), and marbled swamp eel, *Synbranchus marmoratus* (Rocha et al., 2007a) acclimated at 25°C. *(Adapted from Kalinin, A.L., Costa, M.J., Rantin, F.T., Glass, M.L., 2009. Effects of temperature on cardiac function in teleost fish. In M.L. Glass, and S.C. Wood (Eds.). Cardio-Respiratory Control in Vertebrates. Springer-Verlag, Berlin, Germany. pp. 121–160).*

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where R_1 = rate of reaction at temperature T_1 and R_2 = rate of reaction at temperature T_2. Thus, if the rate of a given physiological process subjected to an increase of 10°C in temperature had, at the end of the procedure, a $Q_{10} = 1$, it means that this process is independent of temperature. On the other hand, if Q_{10} is equal to 2 or 3, it means, respectively, that the process rate has doubled or tripled with the increase of 10°C. In most temperate fish species, the f_H increases with a mean Q_{10} of approximately 2 (Priede, 1974; Butler and Taylor, 1975; Cech Jr et al., 1976; Graham and Farrell, 1985; Blank et al., 2002; Tiitu and Vornanen, 2002a). This Q_{10} value also seems to be valid for the f_H of most tropical fish (Rantin et al., 1998). However, some tropical species present Q_{10} values for an f_H of approximately 3 during changes in temperature. This applies to pacu, an active migratory species, which increased f_H from 37 to 115 bpm when the temperature was increased by 10°C (Anelli-Jr et al., 2004). Although a sedentary species, the traíra presented values of Q_{10} near to those of pacu. When in a range of 10°C (from 25°C to 35°C), this species increased f_H from 35 to 105 bpm (Olle, 2003). Interestingly, this also applies to an Antarctic species, *Pagothenia borchgrevinki*, which increased f_H from 16 to 33.7 bpm during a temperature rise of −1°C to 6°C (Lowe et al., 2005), a large increase considering the extremely low ambient temperature. The Q_{10} values for f_H of various species of tropical, temperate, and Antarctic climates are shown in Table 9.2. The data in this table suggest that increases in f_H may signify the best fit triggered for maintaining adequate cardiac performance during increases in temperature. Changes in f_H are considered to be of greatest importance for cardiac output modulations, as shown for tuna fish hearts by Farrell (1996) and Blank et al. (2002). Regarding the low temperatures, Webber et al. (1998) calculated the Q_{10} values for Q and f_H in the Atlantic cod, *Gadus mohua*, under resting conditions. In the thermal range of 5–10°C, the values of Q_{10} were 1.65 for Q and 2.93 for f_H. These authors concluded that a reduction in Q at low temperatures could be attributed to considerable reductions in f_H, as observed in other fish species (Armstrong, 1986; Gehrke and Fielder, 1988; Kolok et al., 1993; Keen and Farrell, 1994; Claireaux et al., 1995).

There is little information available on the effects of temperature on the in vivo cardiac function of fish. Instead, the literature has a considerable volume of information obtained by in vitro studies with perfused hearts (Farrell, 2002). However, few studies have been done on tropical fish, with most of the information coming from data obtained in temperate fish. In vitro studies have shown that in fish, there is an inverse relationship between f_H and *SV* in response to acute changes in temperature (Graham and Farrell, 1985; Webber et al., 1998; Yamamitsu and Itazawa, 1990; Farrell, 2002).

Stevens et al. (1972) studied the cardiac function of the lingcod, *Ophiodon elongatus*, and concluded that in vivo changes in the f_H of this species are more important for the modulation of Q, whereas changes in *SV* are of minor importance. Blank et al. (2002) evaluated the effects of temperature changes on cardiac function in preparations of perfused hearts of yellowfin tuna, *T. albacares*, and observed that f_H dropped linearly from ~106 bpm at 25°C to ~20 bpm at 10°C. Simultaneously, the SV increased with the decrease of temperature, without significant changes in Q. Likewise, the significant reduction in f_H recorded in Atlantic cod when the temperature was reduced from 10°C to 5°C was partially compensated by concomitant increases in *SV* (Webber et al., 1998). Therefore, *SV* adjustments, observed in both in vivo and in vitro studies, are most likely to be more important in Q maintenance when fish face changes in environmental temperature (Farrell et al., 1996).

As discussed above, it is evident that changes in the cardiac function of teleost fish caused by changes in temperature are quite variable (see Tables 9.1 and 9.2). In addition, the methodological differences used in the experimentation, the interspecific characteristics, and the acclimation temperature exert a marked effect on the cardiac parameters.

Matikainen and Vornanen (1992) recorded the f_H of crucian carp, *Carassius carassius*, acclimated at 5°C and 15°C in response to acute temperature increases (Fig. 9.14A). At lower temperatures (2°C to 4°C) the responses were identical, but above this thermal interval, the f_H was higher in fish acclimated to summer (15°C) than in those acclimated to winter (5°C). These authors concluded that this thermal acclimatization, where cold acclimation presents the lowest values of Q_{10}, is the "noncompensatory" or "reverse acclimation" type, according to the classification of Prosser (1973).

Similarly, Maricondi-Massari et al. (1998) found that Nile tilapia, *Oreochromis niloticus*, acclimated to 35°C and subsequently subjected to acute reductions in temperature down to 15°C showed higher f_H at 15 and 20°C than the specimens acclimated at 15°C and subjected to acute temperature rises up to 35°C (Fig. 9.14B and C). On the other hand, Aguiar et al. (2002) observed significantly higher values in the f_H of pacu acclimated to cold (15°C) than those acclimatized to heat (35°C—Fig. 9.14B and C).

Similarly, Morita and Tsukuda (1995) reported that specimens of goldfish, *Carassius auratus*, acclimated to 10°C showed higher f_H than those acclimated at 25°C. When acclimated to heat, the European eel presented lower values of f_H than when acclimated to cold. This difference, however, was abolished by vagal blockade (Seibert, 1979). Similar results were also obtained for medaka, *Oryzias latipes*, guppy, *Lebistes reticulatus* (Tsukuda, 1961), and common sole, *Solea solea* (Sureau et al., 1989).

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TABLE 9.2 Values of Q_{10} for resting heart rate (f_H-bpm) during temperature change in different tropical, temperate, and Antarctic fish species

Species	°C	Q_{10}	Reference
Amboré (tropical) *Bathygobius soporator*	25 to 35	1.6	Rantin et al. (1998)
"Icefish" (Antarctic) *Chaenocephalus aceratus*	2 to −1.8	6.3	Bastos-Ramos et al. (1998)
Atlantic cod (temperate) *Gadus morhua*	5 to 10	2.9	Webber et al. (1998)
Traíra (tropical) *Hoplias malabaricus*	25 to 35	3.0	Olle (2003)
Spangled perch (tropical) *Leiopotherapon unicolor*	5 to 15	1.4	Gehrke and Fielder (1988)
	15 to 25	1.2	
	25 to 35	1.1	
Rockcod (Antarctic) *Notothenia neglecta*	2 to −1.8	4.8	Bastos-Ramos et al. (1998)
Nile tilapia (tropical) *Oreochromis niloticus*	25 to 35	2.4	Costa et al. (2000)
Bald notothen (Antarctic) *Pagothenia borchgrevinki*	−1 to 3	3.3	Lowe et al. (2005)
	3 to 6	2.5	
	6 to 10	1.2	
Pacu (tropical) *Piaractus mesopotamicus*	25 to 35	3.1	Anelli-Jr et al. (2004)
Flatfish (temperate) *Pleuronectes americanus*	4 to 10	2.2	Joaquim et al. (2004)
	5 to 10	1.4	Cech Jr et al. (1976)
Curimbatá (tropical) *Prochilodus lineatus*	25 to 35	2.3	Kalinin et al. (2009)
Marbled swamp eel (tropical) *Synbranchus marmoratus*	25 to 35	1.6	Rocha et al. (2007a)
	25 to 15	2.3	
Yellowfin tuna (temperate/tropical) *Thunnus albacares*	10 to 20	3.6	Blank et al. (2002)
	15 to 25	2.6	
Albacore (temperate/tropical) *Thunnus orientalis*	5 to 15	2.4	Blank et al. (2004)
	10 to 20	2.1	
	15 to 25	1.8	

Adapted from Kalinin, A.L., Costa, M.J., Rantin, F.T., Glass, M.L., 2009. Effects of temperature on cardiac function in teleost fish. In M.L. Glass, and S.C. Wood, (Eds.). Cardio-Respiratory Control in Vertebrates. Springer-Verlag, Berlin, Germany, pp. 121–160.

Based on this information, it can be concluded that cardiac responses to temperature changes are not uniform between teleosts and the effects of thermal acclimatization in f_H are species-specific, reflecting seasonal changes induced in the sensitivity of pacemaker cells (Kalinin et al., 2009).

As in other vertebrates, the f_H of teleost fish is intrinsically determined by the frequency of depolarization of the pacemaker cells and extrinsically by the autonomic nervous system along with humoral factors (Laurent et al., 1983). The temperature affects both intrinsic and extrinsic components. According to Haverinen and Vornanen (2006), low temperatures cause a decrease in f_H and consequently in Q, particularly in environments with large seasonal temperature fluctuations. Many temperate species such as rainbow trout, perch, *Perca fluviatilis*, European eel, and common sole, *S. solea*, are able to avoid the effects on f_H caused by long exposures to low temperatures. These authors suggest that such compensatory

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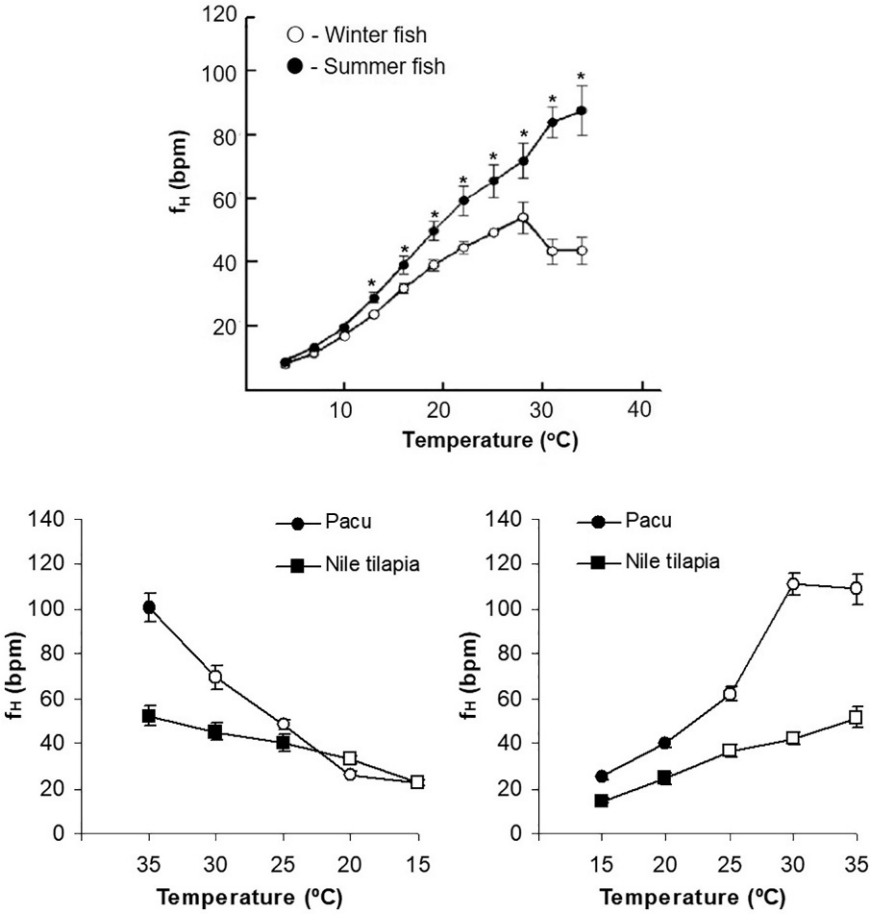

FIG. 9.14 (A) Thermal dependence of in vivo heart rate (f_H) of the crucian carp, *Carassius carassius*, during summer and winter (adapted from Matikainen and Vornanen, 1992). (B) Effect of temperature on heart rate (f_H) of Nile tilapia, *Oreochromis niloticus* (Maricondi-Massari et al., 1998) and pacu, *Piaractus mesopotamicus* (Aguiar et al., 2002) acclimated at 35°C. The empty symbols indicate a significant difference in relation to the initial values *(adapted from Kalinin et al., 2009)*.

mechanisms involve a cold-induced decrease in the cholinergic inhibitory control of the pacemaker. The hearts of teleosts usually operate under a certain degree of vagal tonus, which varies according to environmental and physiological conditions. The magnitude of the action of cardiac-accelerating sympathetic innervation in the fish heart has not yet been adequately elucidated. Some species have adrenergic nerve fibers and an adrenergic tone has been found in vivo, but the relative importance of neural *versus* humoral influences is still unclear. Increases in ventricular contraction force and f_H (positive inotropism and chronotropism) are mediated by β-adrenoceptors in the pacemaker and myocyte cells, whereas α-adrenoceptors modulate negative chronotropism (Egginton et al., 2006).

According to Seibert (1979), in the European eel the vagal tone increases with rises in temperature. This also appears to be the case of pacu, as can be seen in Fig. 9.15B and C (Aguiar et al., 2002). However, in rainbow trout and common carp, the parasympathetic control of f_H predominates at low temperatures while sympathetic control assumes greater importance at higher temperatures (Farrell, 1984). In the Antarctic fish *Pogothenia borchgrevinki*, an increase in f_H during the temperature transition from 1°C to 6°C was due to a 45% increase in excitatory adrenergic tone against a 37% increase in inhibitory cholinergic tone (Lowe et al., 2005). Moreover, Egginton et al. (2006) pointed out that in this species, acclimation to higher temperatures restores the resting f_H to a lower level, indicating an increase in vagal inhibition (Seebacher et al., 2005). These data reinforce the absence of uniform chronotropic responses at the acclimation temperature in teleosts.

Fig. 9.15A shows the effect of temperature on the f_H of pacus acclimated at 15°C and subjected to gradual elevations in temperature up to 35°C and during recovery to the acclimation temperature (upper panel). This figure also shows the effect of temperature on the f_H of fish acclimatized to 35°C and subjected to gradual reductions in temperature down to 15°C and during recovery to the acclimation temperature (lower panel). The electrocardiograms (DI lead of standard electrocardiography) obtained with pacu acclimated at 15°C and submitted to temperatures of 15°C, 20°C, 25°C, 30°C, and 35°C (Fig. 9.15B) show a marked decrease in the RR interval, in the PR and ST segments, in the PR and QT intervals, and in the QRS complex duration as a function of the temperature rise, reflecting the respective chronotropic effects. The details

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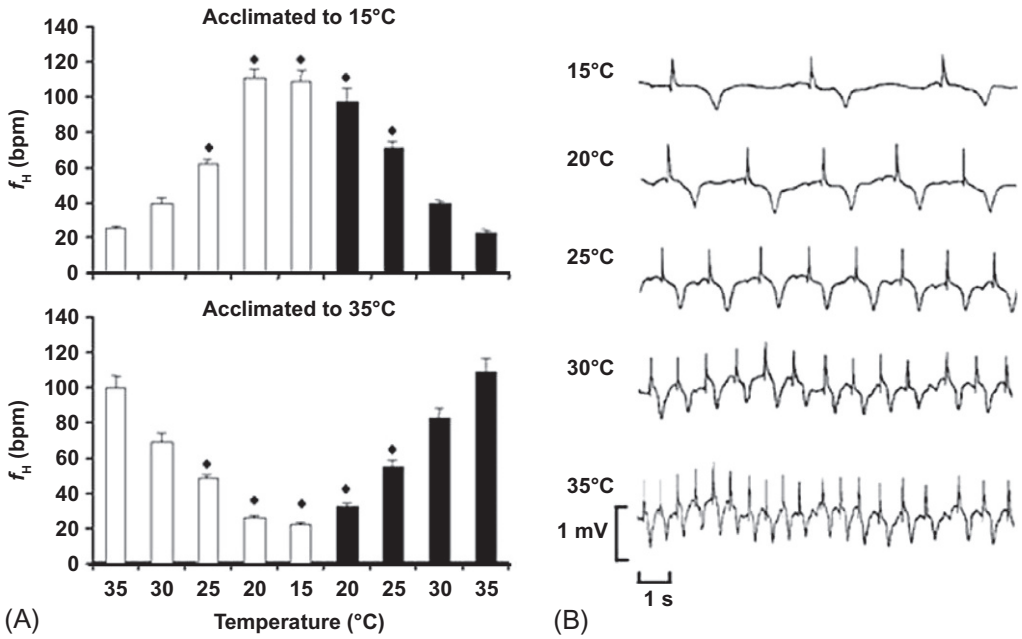

FIG. 9.15 (A) Upper panel: heart rate of pacus, *Piaractus mesopotamicus*, acclimated at 15°C and subjected to gradual elevations in temperature up to 35°C (□) and during recovery following acclimation temperature (▪). Lower panel: acclimatized at 35°C and subjected to gradual reductions in temperature down to 15°C (□) and during subsequent recovery the acclimation temperature (▪). Values expressed as mean ± SEM. * mean significant differences from the initial values. (B) Electrocardiographic recordings (DI lead of standard electrocardiography) of pacu acclimated to 15°C and submitted to temperatures of 15°C, 20°C, 25°C, 30°C, and 3°C. *(Adapted from Aguiar, L.H., Kalinin, A.L., Rantin, F.T., 2002. The effects of temperature on oxygen uptake, gill ventilation and ECG waveforms in serrasalmid fish, Piaractus mesopotamicus. J. Therm. Biol. 27, 299–308).*

of these intervals and segments are depicted in Fig. 9.8. However, no significant change is observed in the morphology of P waves, QRS complexes, and T waves.

In Fig. 9.16, it is observed that the amplitudes of the QRS and T wave complex remained unchanged in both acclimated fish at 15°C and subjected to gradual elevations in temperature up to 35°C and during subsequent recovery to the acclimation temperature as well as in the acclimated ones at 35°C and subjected to gradual reductions in temperature down to 15°C and during recovery to the acclimation temperature. These data indicate that the projection of the sum of the ventricular depolarization (QRS complex) vectors in the electrocardiographic lead line was not altered by the temperature. This implies that the conduction system, within physiological limits, remained constant despite the wide temperature variations. The same can be concluded regarding the direction of ventricular repolarization (T wave). The direction and the sequence of the repolarization of the ventricular myocytes did not undergo any significant alteration due to the thermal alterations to which the fish were submitted.

Similar results were obtained by Morita and Tsukuda (1995) when analyzing the QRS complex of goldfish acclimated at 10°C and 25°C. When subjected to chronic and acute temperature exposures, Nile tilapia presented the same responses as pacu (Maricondi-Massari et al., 1998). Despite these similarities, it is not possible to state that these electrocardiographic findings are common to most teleosts. There are few data available in the literature and more studies are needed to confirm whether there is a pattern of response. Another interesting fact regarding the QRS and T wave amplitudes is that in both pacu and Nile tilapia, these waves were consistently positive, indicating that the depolarization and ventricular repolarization followed the same direction (Maricondi-Massari et al., 1998; Aguiar et al., 2002). The duration of the QRS complex reflects the time required for complete ventricular depolarization while the duration of the T wave represents the time for complete repolarization. According to Fig. 9.17, both QRS and T-wave durations were inversely affected by temperature, that is, when cold-acclimatized fish (Fig. 9.17A) were subjected to gradual elevations in temperature, the durations decreased and vice versa (Fig. 9.17B). These electrocardiographic findings indicate that ventricular depolarization and repolarization are faster at higher temperatures due to its direct effect on pacemaker tissue and AP conduction. A decrease in QRS duration suggests that low temperatures induce a delay in ionic diffusion through the sarcolemma and, as a consequence, the final depolarization. The same effect occurs during the ventricular repolarization process, which is evidenced by the increase in the duration of the T wave.

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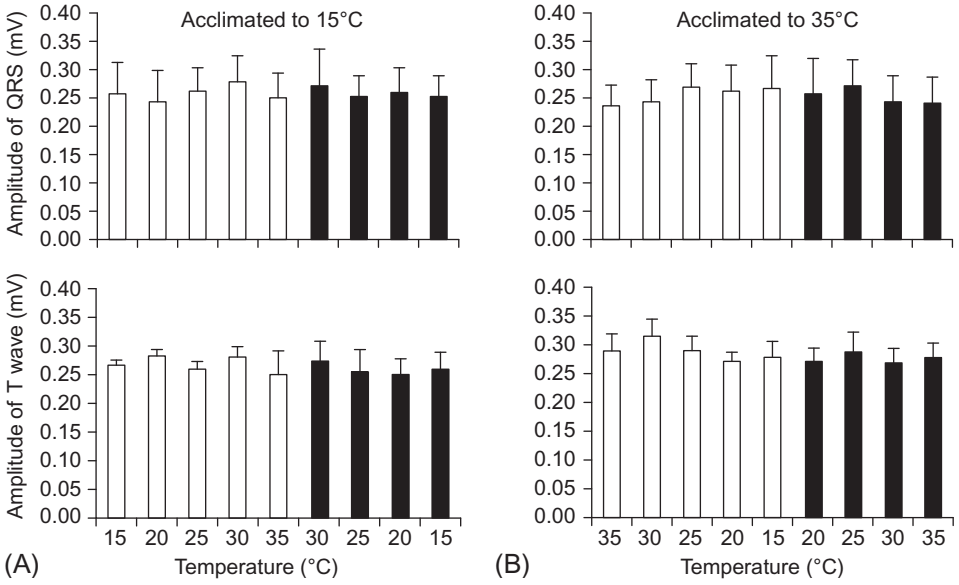

FIG. 9.16 (A) QRS and T-wave amplitudes obtained by electrocardiography in pacu, *Piaractus mesopotamicus*, acclimated to 15°C and subjected to gradual elevations in temperature up to 35°C (□) and during recovery to the acclimation temperature (▪). (B) QRS and T wave amplitudes of electrocardiograms of pacu, *Piaractus mesopotamicus*, acclimated to 35°C and subjected to gradual reductions in temperature down to 15°C (□) and during recovery to the acclimation temperature (▪). Values expressed as mean ± SEM. *(Adapted from Aguiar, L.H., Kalinin, A.L., Rantin, F.T., 2002. The effects of temperature on oxygen uptake, gill ventilation and ECG waveforms in serrasalmid fish, Piaractus mesopotamicus. J. Therm. Biol. 27, 299–308).*

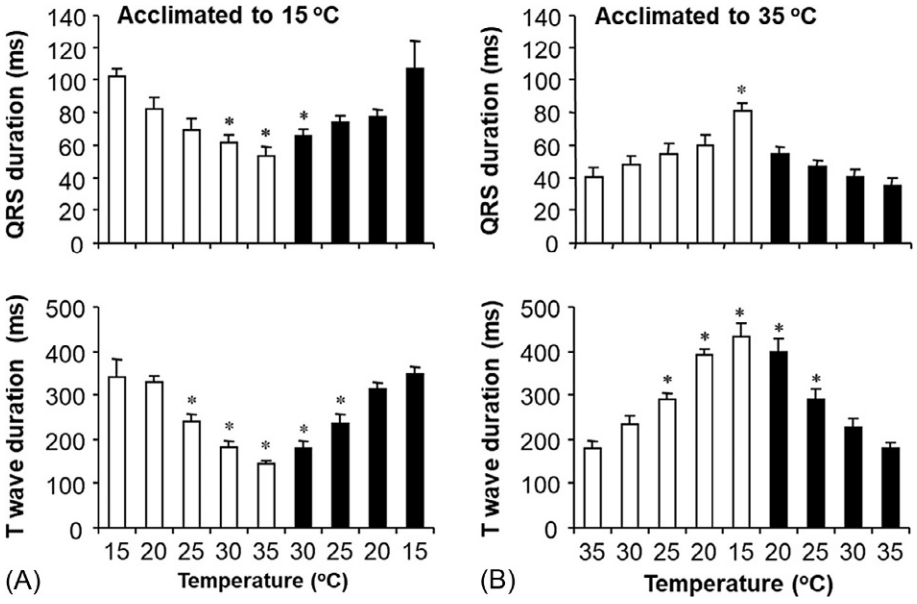

FIG. 9.17 (A) QRS and T wave durations obtained by electrocardiography in pacu, *Piaractus mesopotamicus*, acclimated to 15°C and subjected to gradual elevations in temperature up to 35°C (□) and during recovery to the acclimation temperature (▪). (B) QRS and T wave durations of electrocardiograms of pacu, *Piaractus mesopotamicus*, acclimated to 35°C and subjected to gradual reductions in temperature down to 15°C (□) and during recovery to the acclimation temperature (▪). Values expressed as mean ± SEM. * mean significant differences from the initial values. *(Adapted from Aguiar, L.H., Kalinin, A.L., Rantin, F.T., 2002. The effects of temperature on oxygen uptake, gill ventilation and ECG waveforms in serrasalmid fish, Piaractus mesopotamicus. J. Therm. Biol. 27, 299–308).*

The analysis of the components of the electrocardiographic waves also allows the calculation of the duration of the atrial and ventricular action potentials. The PR and QT intervals (Fig. 9.8B) are, respectively, the durations of atrial AP (A_{APD}—ms) and ventricular AP (V_{APD}—ms). Considering the changes in magnitude, A_{APD} and V_{APD} presented the same trend as QRS and T-wave durations in response to temperature changes, both in cold- and heat-acclimatized pacus (Fig. 9.18). This tendency was also observed in the flatfish, *Platichthys flesus,* (Lennard and Huddart, 1991) and Nile tilapia

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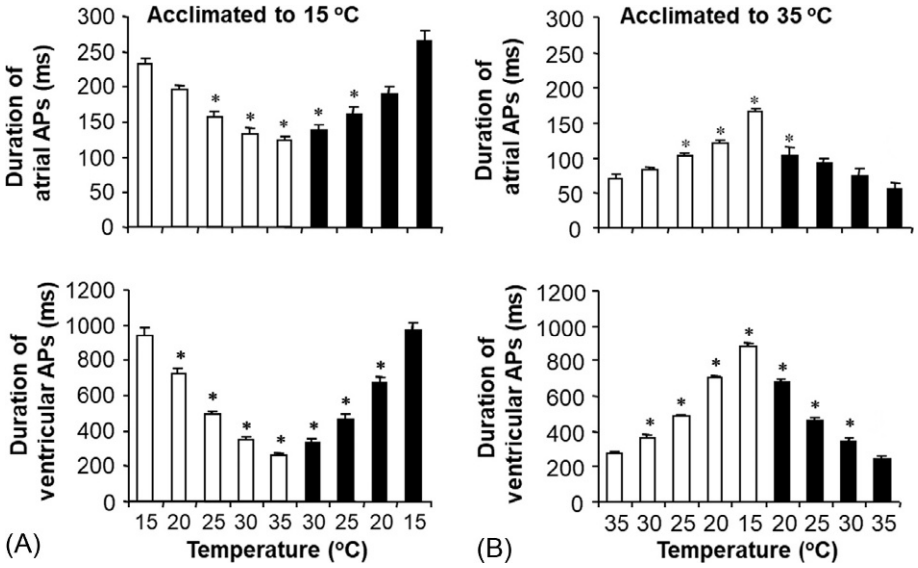

FIG. 9.18 (A) Atrial and ventricular AP duration obtained by electrocardiography in pacu, *Piaractus mesopotamicus*, acclimated to 15°C and subjected to gradual elevations in temperature up to 35°C (□) and during recovery to the acclimation temperature (▪). (B) Atrial and ventricular APs duration obtained by electrocardiography in pacus, *Piaractus mesopotamicus*, acclimated to 35°C and subjected to gradual reductions in temperature down to 15°C (□) and during recovery to the acclimation temperature (▪). Values expressed as mean ± SEM. * mean significant differences from the initial values. *(Adapted from Aguiar, L.H., Kalinin, A.L., Rantin, F.T., 2002. The effects of temperature on oxygen uptake, gill ventilation and ECG waveforms in serrasalmid fish, Piaractus mesopotamicus. J. Therm. Biol. 27, 299–308).*

(Maricondi-Massari et al., 1998), and appears to be common to vertebrate hearts in general (Shattock and Bers, 1987). The effects of temperature variation on AP typical of atrial and ventricular myocytes are shown in Fig. 9.13. According to Vornanen (2017), the electrical excitability of cardiac myocytes depends on temperature changes in order to produce temperature-dependent increases or decreases of heart rate and coordinated changes in the conduction rate of APs to prevent severe cardiac arrhythmias.

The influence of temperature on cardiac contractility, the functionality of ion channels, and potential sources and transport of Ca^{2+} during the excitation-contraction (E-C) coupling of cardiac muscle are of particular interest during temperature changes (Shiels et al., 2002). Acute changes in environmental temperature result in immediate changes in body temperature (Bowler and Tirri, 1990); such changes are not necessarily harmful and may be short-lived. Therefore, the effect of this parameter on cardiac E-C coupling may exert an important influence, especially in the management of Ca^{2+} available for contraction (Vornanen et al., 2002).

Most studies on cardiac contractility in endothermic animals were performed under in vivo conditions and, essentially, at a constant temperature (Hove-Madsen, 1992). Likewise, few studies have addressed the effects of temperature on the management of cardiac Ca^{2+} in tropical fish. Also, information on both acute changes in temperature and adaptations to seasonal variations is quite scarce. Conversely, temperate teleosts, such as rainbow trout and crucian carp, have been extensively studied.

Temperature modifies the rate of enzymatic reactions, including those of cardiac tissue (Marengo et al., 1997). As a consequence, cardiac contractility in fish can be directly influenced by temperature over the duration of the active state, that is, the dynamics of contraction and relaxation (Bailey and Driedzic, 1990). An altered contraction dynamics can modify the total amount of Ca^{2+} activator that binds to troponin C at each contraction, affecting the peak force development. In contrast, temperature-induced changes in the active state may also modify the frequency of contraction, altering cardiac contractility (Shiels et al., 2002).

The ability to tolerate large temperature changes (daily and/or related to water column displacement) is an inherent genetic property that triggers adequate cardiac performance to cope with rapid thermal variations. Therefore, adequate cardiac contractility in response to acute temperature changes is present only in species that have a myocardium with sufficient plasticity to avoid both disturbances in electric excitation and metabolic imbalances (Paajanen and Vornanen, 2004).

Thus, the force of cardiac contraction may increase, decrease, or remain unchanged during an acute change in temperature. For example, ventricular strips of Nile tilapia (Costa et al., 2000), and the goby, *Bathygobius soporator* (Rantin et al., 1998), showed a marked reduction in muscle contraction force during acute elevations of temperature from 25°C to 35°C, despite the fact that such thermal transitions occur in their natural habitat (Fig. 9.19). However, as discussed earlier, both the

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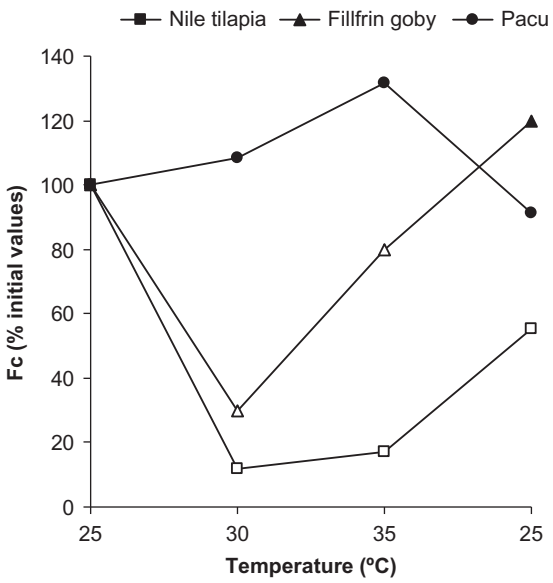

FIG. 9.19 Twitch force (Fc-% initial values) developed by ventricle strips from three species of tropical teleosts acclimated to 25°C and subjected to an acute increase in temperature until 35°C and then to the subsequent return to the acclimation temperature. Open symbols denote a significant difference in relation to the initial values. *(Source: Kalinin, A.L., Costa, M.J., Rantin, F.T., Glass, M.L., 2009. Effects of temperature on cardiac function in teleost fish. In M.L. Glass, and S.C. Wood (Eds.). Cardio-Respiratory Control in Vertebrates. Springer-Verlag, Berlin, Germany, pp. 121–160).*

Nile tilapia and the goby presented increases in the f_H with the elevation of the temperature, being able to increase the Q over a greater frequency of contraction, and not by elevations in the SV. In addition, high temperature-induced chronotropism has a negative inotropic effect due to the typical negative staircase effect of teleost fish (Driedzic and Gesser, 1985; Vornanen, 1989). Nevertheless, pacu presents a marked increase in chronotropism while the inotropic response remains the same (Fig. 9.19) during acute temperature rise from 25°C to 35°C (Anelli-Jr et al., 2004). The marked temperature-induced increase in pacu cardiac output is well correlated with its high activity pattern, as cardiac demand increases proportionally to the acute rise in temperature.

On the other hand, an acute reduction in temperature from 17°C to 2°C did not significantly influence the contraction force of atrial tissue in salmon and trout (Fløysand and Helle, 1994) while the temperature reduction from 20°C to 1°C increased by 60% the contraction force of the crucian carp ventricle (Vornanen, 1989). In these fish, the temperature reduction was associated with a significant increase in the time for the peak force to be reached, which would allow more Ca^{2+} to enter the cell during a single contraction. This response is related to the fact that the AP duration in fish hearts (about 500 ms) is much prolonged when compared to mice hearts (less than 100 ms) (Vornanen, 1989; Møller-Nielsen and Gesser, 1992; Shiels et al., 2006). Contrariwise, an acute increase in temperature decreases the time to force development due to the reduced time available for Ca^{2+} to reach the contractile apparatus during contraction.

The thermal tolerance can be increased by acclimatization because chronic (seasonal) changes in temperature may allow the expression of myosin isoforms or Ca^{2+} transport proteins better adapted to the new thermal environment (Paajanen and Vornanen, 2004). Additionally, there is a direct correlation between these responses and the thermal history of the animal as well as its activity pattern at a given temperature. Therefore, fish need efficient mechanisms for the thermal adaptation of the cardiovascular system (Kalinin et al., 2009).

In short, temperature affects the development of the myocardial contraction force in several ways. High temperatures increase the sensitivity of Ca^{2+} contractile proteins (Harrison and Bers, 1990) and the interval at which cross-bridges remain in maximal force development (Kuhn et al., 1979). Temperature also affects the heart in terms of energy efficiency (Graham and Farrell, 1990), contraction, and cellular Ca^{2+} balance (Bailey and Driedzic, 1990; Lennard and Huddart, 1991). In addition, the relationship between force and contraction frequency is highly temperature-dependent (Hove-Madsen, 1992). This suggests that temperature influences the relationship between contraction and the cellular energetic state of the hearts of teleosts.

Cardiac responses to hypoxia

As aquatic organisms, fish inhabit environments that normally have 30 times less dissolved oxygen than atmospheric air. In addition, aquatic environments such as lakes, marginal lagoons, tidal pools, and other small bodies of stagnant water are

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often subject to reductions in dissolved oxygen concentrations, the environmental hypoxia. The vast majority of teleost species respond reflexively to this hypoxia with hyperventilation, increases in both respiratory rate and ventilatory amplitude, and, concomitantly, bradycardia. It was supposed that in temperate fish, this reflex bradycardia would be induced by the activation of externally oriented oxygen-sensitive gill receptors (O_2 chemoreceptors) monitoring the O_2 concentrations of water from the ventilatory current passing over the gills for gas exchange (Randall and Smith, 1967; Saunders and Sutterlin, 1971; Smith and Jones, 1978; Smatresk et al., 1986; Burleson and Smatresk, 1990a; McKenzie et al., 1991; Burleson and Milsom, 1993; Sundin et al., 2000). On the other hand, in hypoxic-tolerant tropical fish, this bradycardia would be reflexively induced by the activation of internally oriented O_2 chemoreceptors monitoring arterial blood O_2 concentrations (Sundin et al., 1999; Sundin et al., 2000). In this way, hypoxia-tolerant species would adjust heart function in response to arterial hypoxemia while less-tolerant species would respond more quickly to environmental hypoxia. However, recent studies have shown that this hypothesis was not supported, as will be explained below.

The distribution of these O_2 chemoreceptors between the different gill arches is also not uniform among the different species. For example, in Atlantic cod (Fritsche and Nilsson, 1989), rainbow trout (Daxboeck and Holeton, 1978; Smith and Jones, 1978), coho salmon, *Oncorhynchus kisutch* (Smith and Davie, 1984), and traíra (Sundin et al., 1999), these receptors are located in the first pair of gill arches. In the channel catfish, *Ictalurus punctatus*, these receptors appear in the first three pairs of gill arches (Burleson and Smatresk, 1990b) while in the trairão (Micheli-Campbell et al., 2009), jeju, *Hoplerythrinus unitaeniatus* (Lopes et al., 2010), and pacu (Florindo et al., 2006), O_2 chemoreceptors are distributed in the four pairs of gill arches. In tambaqui, *Colossoma macropomum*, besides being present in the four gill arches, these O_2 chemoreceptors also appear in extrabranchial sites (Sundin et al., 2000; Milsom et al., 2002; Florindo et al., 2006). Table 9.3 shows the locations, innervations, and orientations of O_2 chemoreceptors related to cardiac function in the main Neotropical fish species studied to date. In this table, we can observe that even phylogenetically very close species such as traíra, trairão and jeju (Erytrhinidae), tambaqui, and pacu (Characidae) present distinct patterns of localization, orientation, and innervation.

The bradyicardic response is also quite variable among the different species. For example, tambaqui presents a reduction of approximately 63% in the f_H in response to hypoxia (Sundin et al., 2000; Leite et al., 2007) while bowfin, *Amia calva*, responds to hypoxia with a reduction of only 15%. This species does not present gill chemoreceptors associated with cardiac responses, only a population of externally oriented chemoreceptors related exclusively to ventilatory responses (McKenzie et al., 1991). Therefore, it can be concluded that there is no pattern of distribution and orientation of these chemoreceptors among teleosts and the magnitude of bradycardia is also quite variable among fish species under hypoxic conditions.

Gill O_2 chemoreceptors are innervated by the cranial nerves IX (glossopharyngeal) and X (vagus). The IX innervates the first pair of gill arches while the first branch of the X innervates the first and second pairs of arches. The second branch of the X innervates the second and third pairs of arches. The third branch of the X innervates the third and fourth pairs of arches

TABLE 9.3 Locations, innervations, and orientations of O_2 chemoreceptors related to cardiac function in the main Neotropical fish species studied so far

Species	Location	Innervation	Oriention	Reference
Trairão, *Hoplias lacerdae*	4th gill arch	IX and X	Internal and external	Micheli-Campbell et al. (2009)
Traíra, *Hoplias malabaricus*	1st gill arch Extrabranquial	X and possibly IX	Internal	Sundin et al. (1999)
Jeju, *Hoplerythrinus unitaeniatus*	4th gill arch	IX and X	Internal and external	Lopes et al. (2010)
Tambaqui, *Colossoma macropomum*	4th gill arch Extrabranquial	IX and X	Internal and external External	Sundin et al. (2000)
Pacu, *Piaractus mesopotamicus*	4th gill arch	IX and X	Internal and external	Florindo et al. (2006)

Adapted from Micheli-Campbell, M.A., Campbell, H.A., Kalinin, A.L., Rantin, F.T., 2009. The relationship between O_2 chemoreceptors, cardio-respiratory reflex and hypoxia tolerance in the neotropical fish Hoplias lacerdae. Com. Biochem. Physiol. A 154, 224–232.

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and the fourth branch of the X innervates the fourth gill arch as well as the heart and viscera. Fig. 9.20 shows the innervation of the four gill arches of tambaqui by the cranial nerves IX and X.

Although reflex bradycardia is a common response of teleosts to hypoxia, there is no consensus on its physiological significance. According to Farrell (1984), bradycardia would increase the heart filling time, facilitating the O_2 extraction from the venous blood by the internal myocardium. Bradycardia could also increase the efficiency of gas exchange in the gills through the development of adequate cardiorespiratory synchrony (Randall, 1982; Fritsche and Nilsson, 1989; Taylor et al., 1999; Campbell and Egginton, 2007) or even be a protective mechanism for the heart muscle, which is highly aerobic (Taylor et al., 1999).

In fish, the isometric force production of the ventricular muscles deteriorates more rapidly in the trout, which is sensitive to hypoxia, than in the eel, resistant to hypoxia (Hartmund and Gesser, 1992, 1996). The variability in the contractile response may reflect phylogenetic differences in the energy supply/demand ratio of the cardiac muscle, or in the strategy that different species use to overcome the hypoxic period.

According to Vornanen and Tuomennoro (1999), the maintenance of a high cardiac activity requires reinforcement on the production of glycolytic energy, which can result in a risk of metabolic intoxication of the tissue and in the exhaustion of cardiac energy reserves. Instead, depression of cardiac contractility decreases the energy demand of the heart but establishes strict limits on the animal activity.

Due to the reduced energy performance of glycolysis, which represents only a small fraction of the ATP produced by an aerobic metabolism, an adjustment between energy production and energy-consuming processes becomes increasingly important with the prolongation of the hypoxia period.

Vornanen and Tuomennoro (1999) also emphasized that adjustment of cardiac function for the limited availability of metabolic energy may occur by detecting the oxygen content of the environment by peripheral chemoreceptors and subsequent neuromediated inhibition of cardiac contractility, or by metabolites produced locally such as adenosine. Complete depletion of oxygen content in tissues can directly modify contractile function by disrupting intracellular Ca^{2+} homeostasis of cardiac myocytes. Also, according to these authors, the inhibitory vagal reflex, mediated by muscarinic cholinergic receptors, induces hypoxic bradycardia in several species of fish and reduces the contractility of atrial tissue in some

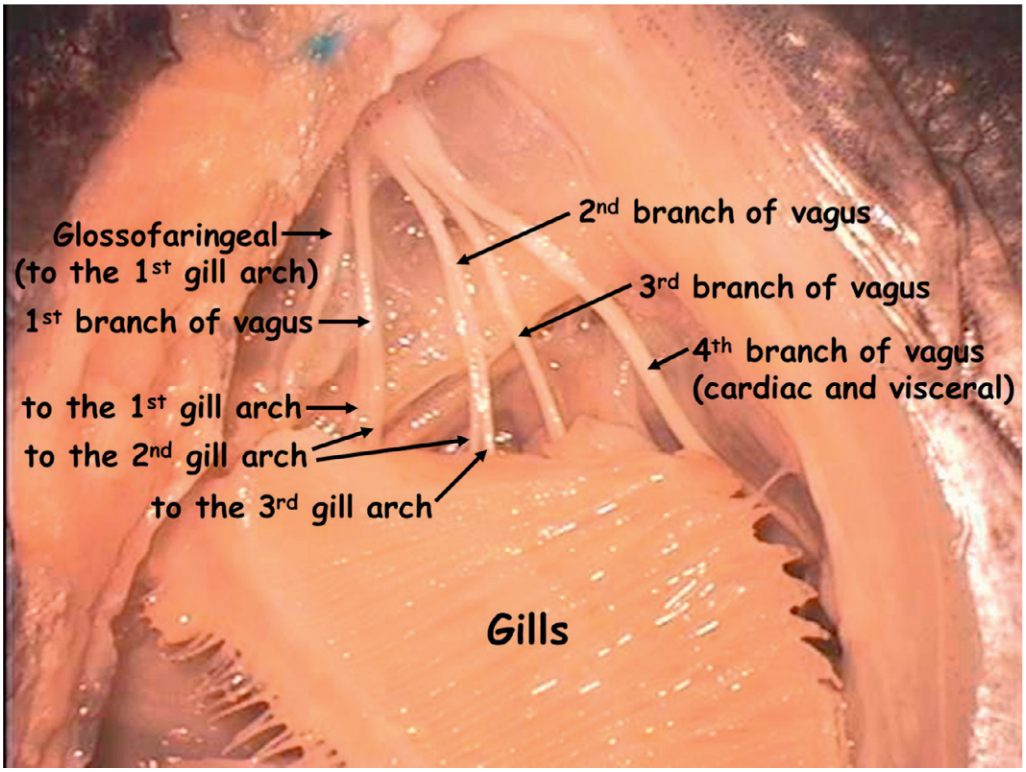

FIG. 9.20 Innervation of the gill arches of tambaqui, *Colossoma macropomum*, by the cranial nerves IX and X. *(Adapted from Florindo, L.H., 2002. O papel dos quimiorreceptores de O_2 e CO_2/pH no controle dos reflexos cardio-respiratórios e da respiração na superfície aquática (ASR) em tambaqui, Colossoma macropomum: Respostas a longo prazo durante a hipóxia e a hipercarbia. Tese de Doutorado. Programa de Pós-Graduação em Ciências Fisiológicas, Universidade Federal de São Carlos, São Carlos, SP, Brasil).*

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teleosts. Purinergic control of the heart is well characterized in mammals, but little is known in fish. In mammals, adenosine tends to balance cardiac function under limited energy conditions, reducing heart work and maintaining energy supply to the myocardium through glycolysis, stimulating cellular glucose uptake (Mubagwa et al., 1996). Although purinergic and cholinergic systems have their own receptors on the sarcolemma of cardiac myocytes, the two systems converge at the level of the sarcolemmal ion channels by the coupling of a specific class of rectifying K^+ channels. The activation of these channels generates an influx current of K^+ that shortens the APs, attenuating the influx of Ca^{2+} by the sarcolemma and, consequently, the contraction force (Belardinelli et al., 1995). However, the relationship between sarcolemmal Ca^{2+} influx and contractile decline during O_2 deprivation remains obscure and the literature lacks data on the effect of hypoxia on myocardial contractility of fish (Driedzic and Gesser, 1994).

Cardiac responses to pollutants

Pollution of the aquatic ecosystems and its toxic effects on aquatic species have been of long-term interest and concern. Contamination of water resources can have a major impact on aquatic ecosystems by causing direct or indirect damage to the associated biota and compromising the health and survival of exposed organisms (Cajaraville et al., 2000). Pollutants penetrate and diffuse into aquatic ecosystems by various routes, including discharge from industrial effluents, agricultural drainage processes, accidental chemical waste spills, and domestic sewage, among others. Thus, aquatic ecosystems are subject to contamination by a wide range of toxic agents such as metals, agrochemicals and pesticides, industrial solvents, flame retardants, and pharmaceuticals, among many other contaminants; these can be incorporated by invertebrate and vertebrate tissues.

The ability of the cardiac muscle to maintain its performance against different physiological conditions is one of the most important characteristics that allows vertebrates to survive in extreme conditions (Driedzic and Gesser, 1994). In fish, as in all other vertebrates, the cardiovascular system is critical to performance and survival because it ensures the effective distribution/transport of oxygen and nutrients to the tissues (Gamperl and Driedzic, 2009). Adjustments in cardiac output in response to pollutants, performed by changes in blood volume and/or heart rate, are extremely important, where cardiac volume is determined by the regulation of myocardial contractility, which depends on the intracellular regulation of calcium in each heartbeat.

According to Incardona and Scholz (2017), as a nonstop pump driven by physiologically complex excitable cardiomyocytes, the heart is particularly susceptible to a variety of insults, mainly xenobiotics. Many pollutants have cardiotoxic effects for fish. Fig. 9.21 shows the effects of organophosphorus insecticides and metals on the development of the myocardial contraction force and the cardiac pumping capacity of different Neotropical fish species. Acute contaminations (96 h) with environmentally relevant concentrations of trichlorfon organophosphates (Thomaz et al., 2009) and methyl parathion (Olle, 2007) induced significant reductions in about 32% and 65% of the myocardial contraction force of tilapia (*Oreochromis niloticus*) and matrinxã (*Brycon amazonicus*), respectively. In these same species, acute and sublethal exposures to metals such as copper (Waldemarin, 2009) and mercury (Monteiro et al., 2017) caused drastic reductions in cardiac output (~55% and 48%, respectively), leading to a loss of heart performance that could reduce cardiac output. Copper also induced significant changes in the cardiomyocytes of Nile tilapia, including cellular and mitochondrial swelling and damages to mitochondrial inner membranes, harming the ATP supply and reducing myocyte contractility (Waldemarin et al., 2012).

Mercury (Hg) is one of the most hazardous environmental pollutants due to its ability to contaminate entire water streams from remote trace level inputs that bioaccumulate through the food chain (Fulkerson et al., 2007). Monteiro et al. (2013) evaluated the effects of trophic and subchronic exposure to inorganic mercury on myocardial contractility and the electrocardiogram (ECG) of traíra (*Hoplias malabaricus*), a widespread sedentary native species that inhabits highly impacted lake systems as a top predator positioned at the highest trophic levels in the food chain. Hg decreased myocardial twitch force development, harmed contraction/relaxation dynamics, and reduced the relative contribution of the Ca^{2+} stored in the sarcoplasmic reticulum (SR) to the E-C coupling. Trophic doses of Hg also induced a marked bradycardia and impaired electrical conduction across the heart, causing a first-degree atrioventricular block (prolongation of the PR interval), lengthening the plateau phase of action potential duration (increased ST segment), and increasing the duration of the atrial and ventricular action potential (increased P and QRS duration).

When fish are exposed to a variety of different chemicals (cyanide, DDT, dieldrin, organophosphorous insecticides, oil dispersant, zinc), a slowing of the heart occurs simultaneously with an increase in ventilation because most of these chemicals also produce internal hypoxia (Heath, 2000). Thus, by slowing the blood flow rate through the gill due to bradycardia response, better oxygenation of the blood could occur.

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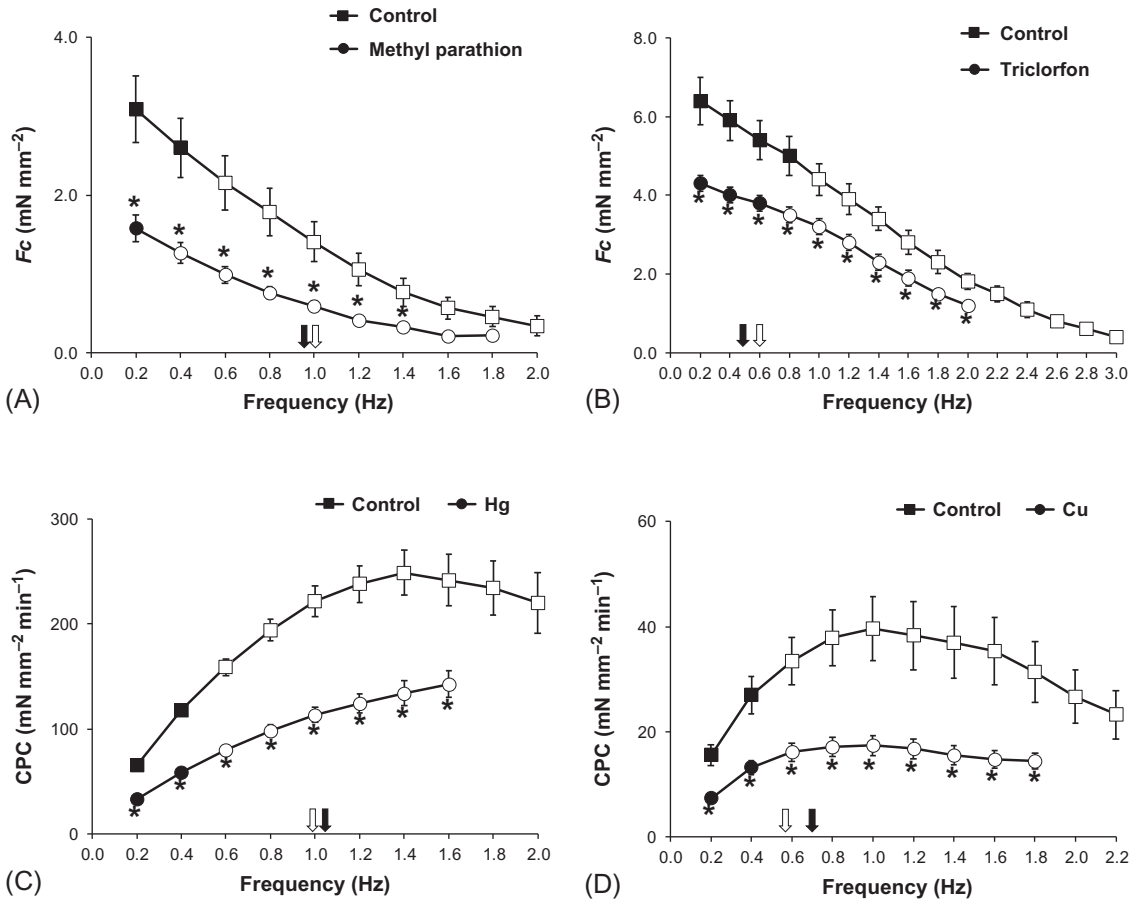

FIG. 9.21 Twitch tension (Fc-A and B) and cadiac pumping capacity (CPC-C and D) developed by ventricular strips from specimens of matrinxã, *Brycon amazonicus* (A and C) and Nile tilapia, *Oreochromis niloticus* (B and D) at different stimulation frequencies after acute (96 h) and sublethal exposures to organophosphate compounds (2.0 mg L^{-1} of methyl parathion and 0.5 mg L^{-1} of trichlorfon) or to metals (1 mg L^{-1} of $CuSO_4$ and 0.1 mg L^{-1} of $HgCl_2$). The white and black arrows indicate the heart rate measured in vivo to control and exposed fish, respectively. Mean values ± SEM. Open symbols denote a significant difference in relation to the values obtained at 12 bpm while asterisks indicate significant differences between experimental groups at the same frequency. *(Sources: Olle, C.D., 2007. Efeito do inseticida organofosforado metilparation (Folisuper 600BR) sobre a função cardio-respiratória do peixe teleósteo matrinxã, Brycon cephalus. Tese de Doutorado. Programa de Pós-Graduação em Ciências Fisiológicas, Universidade Federal de São Carlos, São Carlos, SP, Brasil; Waldemarin, K.C.A., 2009. Efeito do sulfato de cobre sobre a função cardíaca da tilápia tailandesa, Oreochromis niloticus niloticus. Tese de Doutorado. Programa de Pós-Graduação em Ciências Fisiológicas, Universidade Federal de São Carlos, São Carlos, SP, Brasil; Monteiro, D.A., Taylor, E.W., Rantin, F.T., Kalinin, A.L., 2017. Impact of waterborne and trophic mercury exposures on cardiac function of two ecologically distinct Neotropical freshwater fish Brycon amazonicus and Hoplias malabaricus. Comp. Biochem. Physiol. C 201, 26–34; Thomaz, J.M., Martins, N.D., Monteiro, D.A., Rantin, F.T., Kalinin, A.L., 2009. Cardio-respiratory function and oxidative stress biomarkers in Nile tilapia exposed to the organophosphate insecticide trichlorfon (NEGUVON). Ecotoxicol. Environ. Saf. 72(5), 1413–1424).*

The teleost fish embryos are extremely sensitive to low levels (ng L^{-1}) of petroleum hydrocarbons (polycyclic aromatic hydrocarbons, PAHs). These induce edema and change contractility and heart rate, which impair swimming performance (Cherr et al., 2017). The disruption of cardiac function is induced by a diverse array of toxic chemical contaminants in freshwater, estuarine, and marine habitats, negatively affecting fish survival at hatching as well as later life stages (Incardona and Scholz, 2016).

Furthermore, the pollutants can modify the gene expression of cardiac ion channels, pumps, and exchangers. Atlantic haddock (*Melanogrammus aeglefinus*) embryos exposed to crude oil displayed reduced NCX expression that regulates intracellular calcium levels during E-C coupling (Sørhus et al., 2016). Phenanthrene (5 μM) disrupted Ca^{2+} dynamics and impaired Ca^{2+} transient amplitude and decay rate in ventricular myocytes from Pacific mackerel (*Scomber japonicas*), yellowfin tuna (*Thunnus albacares*), and Pacific bluefin tuna (*Thunnus orientalis*) (Brette et al., 2017). Disruption of intracellular Ca^{2+} levels reduced heart contractility and led to a reduced stroke volume, cardiac output, arterial blood pressure, and tissue perfusion.

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Subtle cardiotoxic effects can cause serious and long-lasting outcomes. Pollutants decrease the plasticity of the cardiac responses, reducing the physiological performance and, consequently, the survival chances. Thus, cardiovascular disorders and cardiotoxicity induced by pollutants is a real-world environmental health concern for wild fish populations.

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Chapter 10

Breathing and respiratory adaptations

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Introduction

Respiration or "external respiration" refers to the gas exchange, oxygen (O_2) uptake, and carbon dioxide (CO_2) release between the environment and blood through the surface of respiratory organs. Cellular respiration or "internal respiration" refers to the O_2 metabolism for energy production. During this process, chemical energy from biomolecules (glucose, lipids, and proteins) is converted into ATP in the presence of O_2 and used in cellular processes; as the result of such a metabolism, CO_2 is released from cells. However, cellular respiration will not be discussed in this chapter.

Fish, like other aerobic organisms, require O_2 to maintain energy for metabolic processes. Most of them obtain the O_2 necessary to their metabolic demand directly from the aquatic environment by flowing water through the gills. However, some fish are facultative or obligatory air-breathers and have an accessory respiratory organ to complement their O_2 needs by taking O_2 from atmospheric air.

Aquatic respiration is extremely limited due to the high viscosity of water and the low O_2 solubility as compared to atmospheric air (Dejours, 1981). The development of a unidirectional mechanism to ventilate the gills by moving water from the buccal to opercular cavities and a countercurrent system between the water and blood in the respiratory structures favors gas diffusion across the gill epithelium, resulting in a highly efficient process for O_2 extraction that can reach up to 80% of the aqueous dissolved O_2 in some fish (Fernandes and Rantin, 1989). The body surface plays an important role in the process of gas exchange in the early life stages of development when the gills are not yet fully developed and in species that emerge voluntarily from the aquatic environment. These species include gobies or those in which the environment disappears during the dry season, such as lungfish. The organs for aerial respiration in air breathing have evolved independently in some groups, allowing those fish to survive in stagnant and oxygen-poor environments.

The process of breathing provides O_2 to the tissue metabolism and removes CO_2 produced during the oxidative metabolism. The O_2 consumption and CO_2 production vary depending on the metabolic rate of the animal. Several factors affect the O_2 demand, including individual parameters such as size and activity as well as hydrographic parameters such as water temperature, salinity, and O_2 availability. The degree of activity profoundly alters O_2 consumption and as such, the aerobic metabolism varies within the limits between routine O_2 consumption and the maximum degree of aerobic activity during exercise. In fish, temperature is the main factor that affects O_2 demand, due to the close relationship between water temperature and body temperature. Smaller species have higher O_2 consumption per kg body mass than the larger ones. The O_2

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availability (hyperoxia, normoxia, or hypoxia) in the aquatic environment determines the behavioral and physiological respiratory adjustments required to meet the O_2 demand of a given species. Respiratory function is closely linked to cardiac activity and circulation, and adjustments in respiratory variables are associated with adjustments in the cardiovascular system (see chapters on the Cardiovascular System and the Blood and Circulatory System). This chapter will be divided into two parts: the morphofunctional aspects of the gills and accessory organs for air breathing and the respiratory physiology with an emphasis on the adjustments of gill ventilation in response to environmental changes.

Respiratory gases in natural waters

The concentration of gases dissolved in water is more complex than in atmospheric air. Both the physical properties of water and the dynamics of atmospheric gases in aquatic environments are basic elements needed to understand the distribution, behavior, reproduction, growth, and survival of organisms living in these environments.

Water in equilibrium with air has the same partial gas pressure as atmospheric air, but a lower molar concentration of gases. The molar concentration of a gas (G) in water is described by Henry's Law: $[G]=PG\alpha G$, where $[G]$ is the molar concentration of gas ($\mathrm{mol\,L^{-1}}$), PG is the partial pressure of gas, and αG is the solubility coefficient of gas. The solubility coefficient of a gas depends on its nature (O_2, N_2, CO_2, etc.) and the nature of the solvent (water, ethanol, lipids, etc.) as well as temperature, salinity, and other factors. In general, the solubility of a gas decreases with increasing temperature and salinity. For example, at 10°C, one liter of water saturated with air contains 11.3 mg O_2 (0.35 $\mathrm{mM\,L^{-1}}$) and one liter of air at the same temperature and atmospheric pressure contains 296 mg O_2 (9.25 $\mathrm{mM\,L^{-1}}$); at 0°C and 30°C, one liter of water saturated with air contains 14.16 and 7.53 mg O_2 $\mathrm{L^{-1}}$, respectively. At the same temperature, sea water saturated with air contains 20%–30% less O_2 than freshwater. In comparison, CO_2 is 25–30 times more soluble than O_2 (Dejours, 1981). Therefore, the volumes of O_2 and CO_2 in water are not equal to air at the same partial pressure.

In the atmosphere, the partial pressure of O_2 (PO_2) and the concentration of O_2 are approximately constant. However, in aqueous environments, the PO_2 and $[O_2]$ in water can vary drastically. The fluctuations of O_2 and CO_2 in natural waters depend on numerous spatial and temporal factors. For example, diurnal fluctuations of O_2 depend on the balance between photosynthesis (O_2 production by aquatic plants) and respiration (O_2 consumption) within the aquatic environment. These fluctuations are very common, especially in shallow water bodies where the O_2 production in an abundance of plants and light availability increases the O_2 level to above saturation with atmospheric air during the day (hyperoxia) and decreases (hypoxia) it sharply at night due to the continuous breathing of plants and animals. In deepwater bodies and high water volume, the level of O_2 may vary depending on the light penetration. In that case, stratification may occur. In temperate regions, the formation of ice on water bodies during winter prevents O_2 equilibrium with atmosphere and the level of O_2 depends on the photosynthetic activity and the O_2 demand by the community. In tropical regions, the reduction of dissolved O_2 in the aquatic environment can reach very low levels or even total depletion, which may occur from the high temperatures associated with the low solubility of O_2 in water and the O_2 demand for the respiratory activity of aquatic communities.

The pollution of natural waters is another important factor in depleting dissolved O_2. The introduction of organic matter and its decomposition in the aquatic environment generates eutrophication, which can contribute to an increase in the fluctuation in O_2 concentration in these environments because the oxidation of the organic matter by microorganisms depends on the dissolved O_2.

Diffusion of gases and respiration

At any temperature above absolute zero, all molecules have random movement whose velocity increases with an increase in the temperature (Brownian motion). This movement is the mechanism for the diffusion process. Molecule diffusion occurs at random but is directed from one region with a high concentration to another one with a lower concentration following the Second Law of Thermodynamics. The rate of diffusion of a molecule between two points is determined by its size and spatial molecular form, the concentration gradient, and diffusion distance. Fick´s Law for diffusion quantitatively defines the relationships between these three factors for respiratory gases. It postulates that the amount of a diffused molecule per unit time, that is, the diffusion rate (J) is proportional to the diffusion coefficient (D, $\mathrm{cm^2\,s^{-1}}$), the available area (A, $\mathrm{cm^2}$), and the concentration gradient ($[C]_1-[C]_2$, $\mathrm{mol\,cm^{-3}}$), and inversely proportional to the diffusion distance (x):

$$J=DA\left([C]_1-[C]_2\right)/x\ \left(\mathrm{mol\,s^{-1}}\right)$$

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where D is a physical constant expressed by the molar concentration gradient, which is considerably higher in the air than in the water, increases almost proportionally with increasing temperature, and is inversely proportional to the square root of the molecular mass of the solute. The diffusion coefficient for many biological tissues is like that of water. The Krogh diffusion coefficient (K), another common form of D, evaluates the diffusion rate from the partial pressure gradient of gases and not the gradient of molar concentration. The two forms of the diffusion coefficient are interconvertible via the coefficient of solubility (water) or capacitance (air).

The effectiveness of diffusion for gas exchange is suitable for animals of very small size (<1 mm in diameter). Therefore, the development of a mechanism to maintain the flow of water on the respiratory surface and the circulatory system that maximizes the blood flow inside vessels (convection) was essential for animals larger than 1 mm. As such, a specialized large and thin surface was developed for gas exchange, maximizing the O_2 diffusion from water to blood across the respiratory epithelium and ultimately its distribution throughout the body.

Respiratory organs in fish

Gills, skin, and accessory organs for aerial respiration are the gas exchange areas in fish, but the relative amounts of gas exchange vary depending on the stage of the development of the animal and the environmental condition. The gills are, in general, the major organs of gas exchange in most fish, although some species have accessory organs for air breathing. The importance of aquatic respiration for fish having accessory organs for air breathing is associated with the dependence on dissolved oxygen in the aquatic environment or atmospheric air. In both cases, the release of CO_2 occurs mainly in the aquatic environment.

Gills are complex and multifunctional organs that are directly involved in the exchange of respiratory gases (O_2/CO_2), ionic and osmotic regulation, acid-base balance, nitrogenous product excretion, metabolism, and excretion of xenobiotics. Integrating all gill functions implies an "osmorespiratory compromise" that involves adaptation and requires a precise control mechanism (Laurent, 1989). Anatomically, the gills consist of a system of successive subdivisions whose organization is flexible enough to allow fish to inhabit diverse environments and even withstand environmental fluctuations (Laurent and Perry, 1991). The adaptation process involves a morphological component or has morphological consequences. Adaptation implies finding a new state of equilibrium in order to preserve the gill functions. Gill morphology and functions are intrinsically related and undergo constant modifications during fish adaptation to environmental changes so that the optimization of one function may compromise another.

Gills

In recent decades, gills have received special attention from biologists due to increased water pollution. Also, there is a need to better understand the function of the main blood-water interface in fish.

Organization and structure

The pharynx, a region of the anterior digestive system situated between the oral cavity and the esophagus, is the site of the origin and development of the internal gills (Romer and Parsons, 1985). The organization of the gills is relatively simple in Agnates and increases in complexity in elasmobranchs and teleosts (Evans et al., 2005; Hirose et al., 2003). The gills constitute the gill arches, filaments (i.e., primary lamellae), and lamellae (i.e., secondary lamellae) (Fig. 10.1A–D). In this chapter, the terminology of filament and lamella will be used.

On each side of the pharynx, there are four gill arches, which consist of two bones: the epibranchial and the ceratobranchial bones (Fig. 10.1B and C). The anterior region of the gill arches faces the buccal cavity and has rakers, whose structure, shape, and number depend on the feeding habits of the species. In the posterior region, the filaments project into the opercular cavity being cove by the operculum, which forms a single outer opening along the posterior region on each side of the animal (Fig. 10.1A–C).

Two rows of filaments originate in the gill arches and are joined by a continuous common structure, the interbranchial septum, where muscles and nerves are located the interbranchial septum (Fig. 10.2A). Each filament row is called a hemibranch. The anterior hemibranch faces the operculum and the posterior one faces the pharynx; both hemibranchs are called holobranch (Fig. 10.2A and B). In the teleost, a thin cartilage ray supports the filament along its entire length close to the afferent primary artery (aa1,) and is articulated with branchial bones (Fig. 10.2B). The lamellae that are the respiratory units are localized above and below the horizontal axis of each filament (Fig. 10.2B). The gill apparatus has been described in

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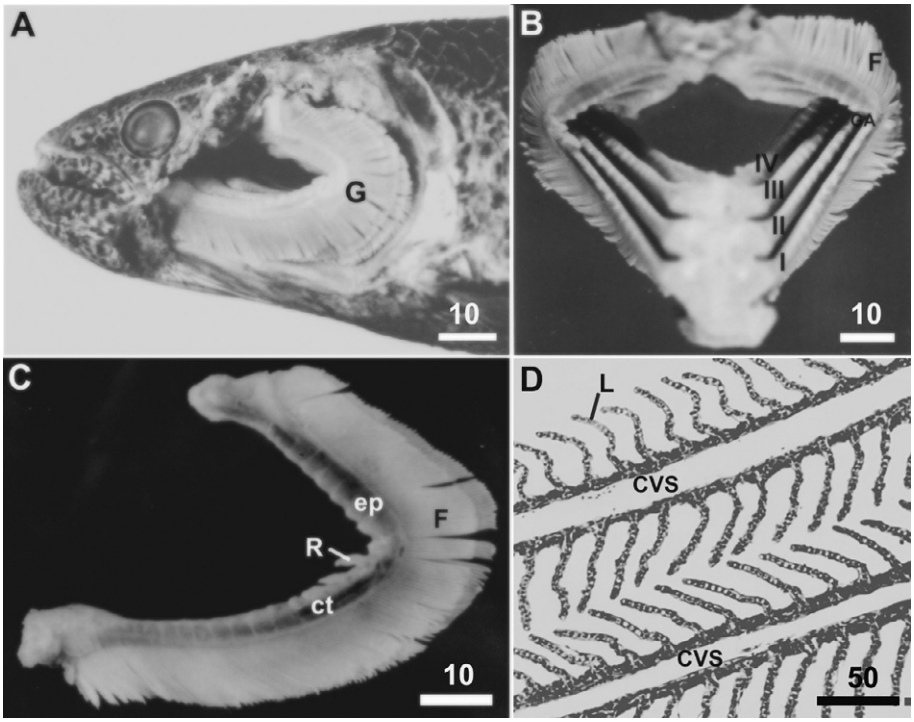

FIG. 10.1 *Hoplias malabaricus*. (A) Lateral view of *Hoplias malabaricus* head showing the gills (G). the operculum was removed. (B) Gill arches (I, II, III, and IV). (C) Gill arch showing rakers and filaments. (D) Gill filaments showing the lamellae above and below its horizontal axis. *GA*, gill arches; *ct*, ceratobranchial bone; *ep*, epibranchial boné; *G*, gills; *F*, filament; *L*, lamella; *R*, raker; *CVS*, central venous sinus. Scale bars = mm in A, B and C; μm in D.

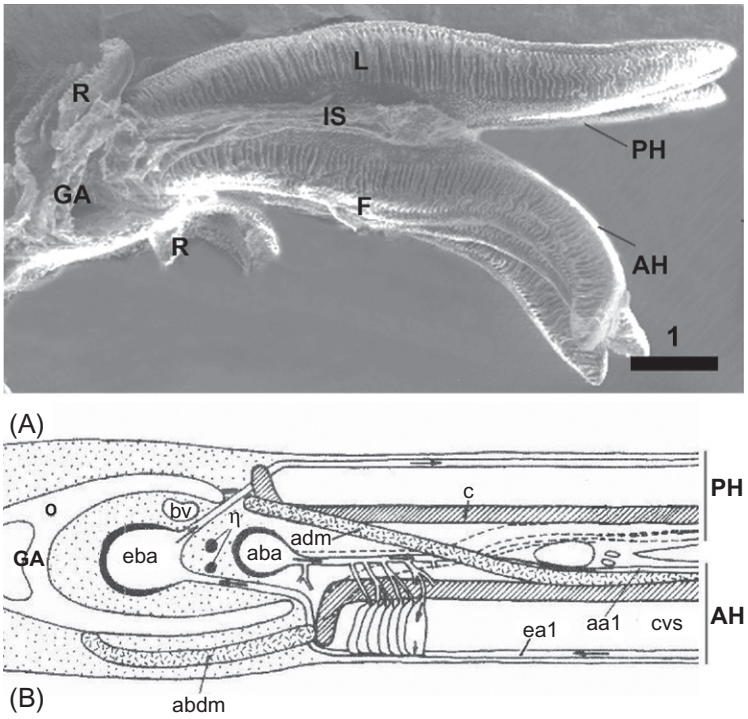

FIG. 10.2 (A) Cross-section of gill arch showing the anterior (AH) and posterior (PH) hemibranchs. *F*, filament; *GA*, gill arch; *IS*, interbranchial septum; *L*, lamellae; *AH*, anterior hemibranch; *PH*, posterior hemibranch; *R*, raker. Scale bars = μm. (B) Schematic of the gill structures. *aba*, afferent branchial artery; *eba*, efferent branchial artery; *aa1*, afferent primary artery; *ea1*, efferent primary artery; *adm*, adductor muscle; *abdm*, abductor muscle; *bv*, branchial vein; *c*, cartilage; *cvs*, central venous sinus; *L*, lamella; *GA*, gill arch; *AH*, anterior hemibranch; *PH*, posterior hemibranch.

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different freshwater fish of the Brazilian ichthyofauna, from elasmobranchs such as the freshwater stingrays of the Amazon region (Duncan et al., 2010, 2015) to different teleost families such as Loricariidae (Cruz and Fernandes, 2016; Fernandes and Perna, 1995; Fernandes et al., 1995b; Santos et al., 1994), Curimatidae (Lima et al., 2009), Prochilodontidae and Sciaenidae (Mazon et al., 1998), Erythrinidae (Fernandes and Moron, 1996; Moron et al., 2009), Serrasalminae (Severi et al., 2000), Characidae and Pimelodidae (Paulino et al., 2014), Arapaimidae (Fernandes et al., 2012; Ramos et al., 2013, 2018), and Dipnoi, which is the single representative of the lungfish in South America, *Lepidosiren paradoxa* (Moraes et al., 2005; Wright, 1974).

The length of the interbranch septum varies in different groups of teleosts. Dornesco and Miscalenco (1968) distinguished three interbranch septum types, studying more than 50 species distributed in 56 orders. The clupeiform type has a long septum that accompanies two-thirds of the filament length and there is communication between the aa1, the cypriniform has a septum which length extends to approximately ½ of the filament length and there is also a communication between the aa1. In the perciform type, the septum is very short and there is no communication between the aa1. In the elasmobranchs, the interbranchial septum exceeds the filament length and forms the external gill slits.

The abductor and adductor muscles are striated, playing an important role in the respiratory cycle; their arrangements vary in different groups (Fernandes et al., 1995b; Hughes, 1984). The abductor muscle has fibers connecting the gill bones (epi- or ceratobranchial) to the cartilage ray of the filament (Figs. 10.2B and 10.3A–C). The fibers of the adductor muscle originate at the base of the filament close to the cartilage ray of a hemibranch; they attach to the cartilage of the filament of the opposite hemibranch (Figs. 10.2B and 10.3A–D). In the terminal portion of the interbranchial septum, bundles of smooth muscle fibers and collagen fibers bind the filament cartilages of the same hemibranch. The organization of the adductor muscle was described in detail by Bijtel (1949), who distinguished two types of adductor muscles. In type I, the muscle of the filament of a hemibranch crosses the interbranchial septum and fixates in the cartilage of the opposite hemibranch, as in the tropical fish Nile tilapia, *Oreochromis niloticus* (Fig. 10.3A). In type II, muscle bundles are located horizontally along the length of the filament (intrafilamental) and inserted in a ligament composed of elastic fibers in the opposite hemibranch in the distal region of the interbranchial septum, as observed in the Erythrinidae, *Hoplias malabaricus* (traíra), *Hoplias lacerdae* (trairão), *Hoplerythrinus unitaeniatus* (jeju), and the Hypophthalmidae, *Hypophtalmus edentatus* (mapará) (Fig. 10.3B). Furthermore, Fernandes et al. (1995b) described a third type of adductor muscle in the Loricaridae family. *Hypostomus tietensis* (catfish) and *Rhinelepis strigosa* (black catfish) have transverse and oblique bundles of

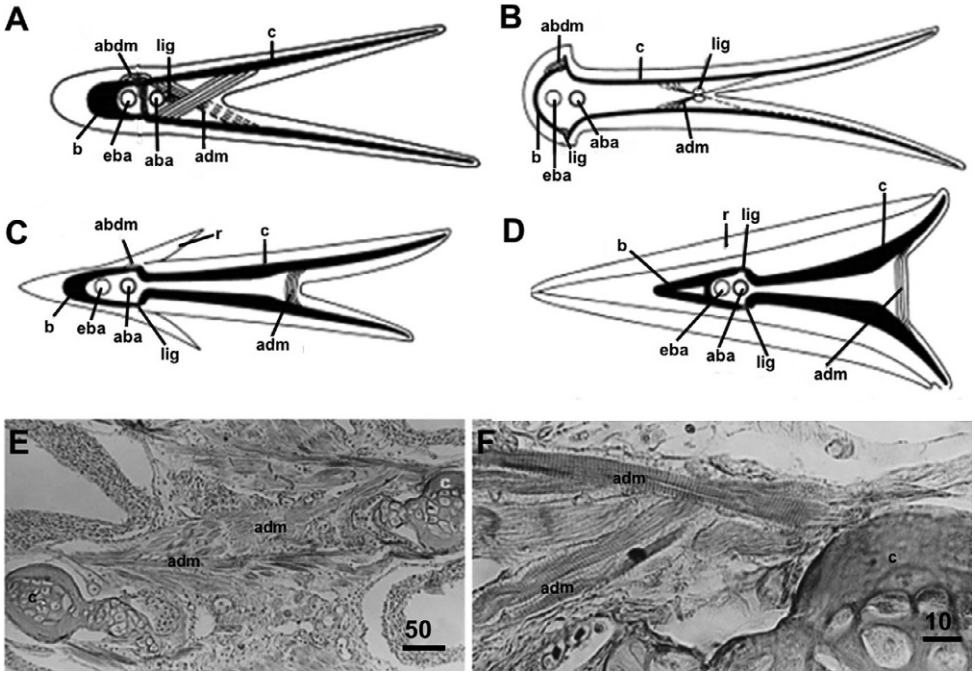

FIG. 10.3 (A–D) Schematics of the filament abductor (abdm) and adductor (adm) muscles of teleost. (A) Adductor muscle-type I; (B) Adductor-type II; (C) and (D) Adductor muscle-type III in *Hypostomus* (C) and *Rhinelepis* (D). (E) Photomicrograph of cross-section of interbranchial septum of *Hypostomus* showing the adductor muscle (adm). (F) Detail of the insertion of the adductor muscle (adm) in the cartilage ray of the filament. *Aba*, afferent branchial artery; *eba*, efferent branchial artery; *c*, cartilage ray; *lig*, ligament; *b*, gill arch bone; *r*, raker. Scale bar = μm. *(Photo: S.E. Moron.)*

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striated muscles in the distal region of the interbranch septum that attach to the cartilage of filaments of opposing hemibranchs, allowing for two types of movements in order to approximate the filaments of the same and different hemibranches (Fig. 10.3C–F). In *Rhinelepis*, the abductor muscle is absent (Santos et al., 1994) and in *Hypostomus* it consists of a small number of fibers (Fernandes et al., 1995b).

Gill circulation consists of an artery-arterial system, sometimes referred to as respiratory circulation, that is directly associated with gas exchange, and an arteriovenous system that irrigates the other structures of the filament and is associated with ionic regulation (for details, see Hughes, 1984, Laurent, 1984). The artery-arterial system consists of the afferent branchial artery (aba) located in the branchial arch, which leads to the aa1 at each insertion of the filament. The aa1 extends to the free apical end of the filament, giving rise to the afferent secondary arteries (aa2) or arterioles that supply the lamellae (Figs. 10.2B and 10.4A–B). In the lamella, blood flows between the spaces formed by the extensions of

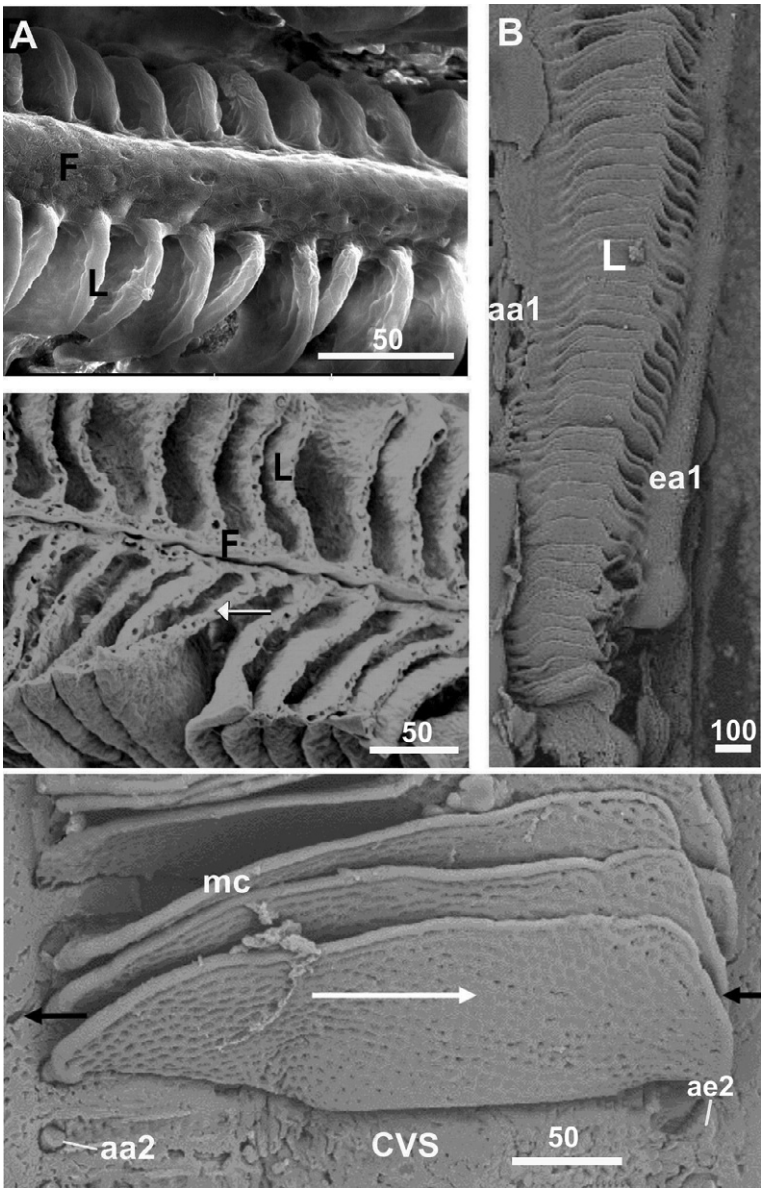

FIG. 10.4 Scanning electron photomicrograph of gill filament and lamella. (A) Filament (F) of *P. maculatus* showing the lamellae (L). (B) Vascular cast of filament and lamellar circulation. (C) Detail of filament and lamella showing the spaces formed (arrow) by the pillar cells flanges in which the blood flows. (D) Vascular cast of a lamella showing marginal channel (mc) and blood spaces. Black arrows indicate the water flow between lamellae and the white arrow indicates the blood flow in the spaces formed by the flanges of pillar cells. *aa2*, afferent secondary artery; *CVS*, central venous sinus. Scale bar = μm. *(Photo: S.E. Moron.)*

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the pillar cells, the proximal and marginal channels, in a countercurrent to the water flow in the interlamellar space, allowing for gas exchange (Figs. 10.2B and 10.4C–D). Oxygenated blood flows through the secondary efferent arteries (ea2) and supplies the efferent primary arteries (ea1) in the filament (Figs. 10.2B and 10.4C–D). The ea1 from each filament forms the branchial efferent artery (eba) in the branchial arch (Fig. 10.2B). The ebas of the four gill arches form the dorsal aorta. The arteriovenous system is distributed in the central venous sinus, which constitutes the internal region of the filaments between the aa1, cartilage, and ea1 and extends along the filament. The arterio-venous system consists of a nutritive vascular system and arterio-venous anastomoses. Nourishing blood vessels have their origins in eba and ae1, and anastomoses are mainly derived from ea1. From the central venous sinus, blood is drained into the branchial vein and returns to the heart. An interesting aspect in the circulation of loricariid fish was described by Fernandes and Perna (1995) and Santos et al. (1994) in *Hypostomus* and *Rhinelepis*. In the median region of the interbranchial septum of species within this genus, there is an interconnection between the aa1s of opposite hemibranchs and between adjacent filaments. The aa1s practically disappear and the blood circulates between numerous endothelium-coated trabeculae containing smooth muscle and collagen. In the distal region of the septum, the aa1s appear again and can be individually identified in each filament extending until the free portion of the filaments. However, the function of these anastomoses still needs to be clarified.

Gill circulation is directly associated with blood volume, the pressure generated by cardiac function, and the changes in systemic and branchial vascular resistance. These physical factors may: (1) shift blood flow from the arterial-arterial system to the central venous sinus via the arteriovenous anastomosis; (2) change the number of perfused lamellae; or (3) distribute the blood flow throughout the lamella or restrict the blood flow only to the proximal and marginal channels.

The lamellae are regularly distributed along the length of the filament; the water flows in the space between lamellae (Figs. 10.2A and 10.4A). The lamella constitutes pillar or pilaster cells whose cytoplasmic extensions form channels through which blood circulates (Fig. 10.5A and B). The pillar cells contain filaments of actin and myosin; the contractions of these filaments alter the volume of the space formed by the extensions of these cells. The proximal (filament-embedded) and marginal (apical lamellae) channels consist of endothelial cells and extensions of the pillar cells (Figs. 10.4C and 10.5A). These two channels generally have larger diameters than those formed from extensions only by pillar cells and present less resistance to blood flow (Fig. 10.5A). The lamellar epithelium and basement membrane cover the pillar cells (pillar cells + blood spaces) (Fig. 10.5B). The gas exchange occurs in a countercurrent system between the blood and water flow.

The gills of most teleost have a basic morphology, geometrically arranged in filaments and lamellae. However, there is some variation in fish that have accessory air-breathing organs. Among them, the lungfish *L. paradoxa* (pirambóia) has very small gills organized as papilla-like filaments (Fig. 10.6A–C) (Moraes et al., 2005). The pirarucu, *Arapaima gigas*, an obligatory air-breather species, has gills like other teleosts up to approximately 200 g. As the fish grows, the gills undergo profound modifications such as increased filament and lamella epithelium thickness and atrophy of pillar cells in the

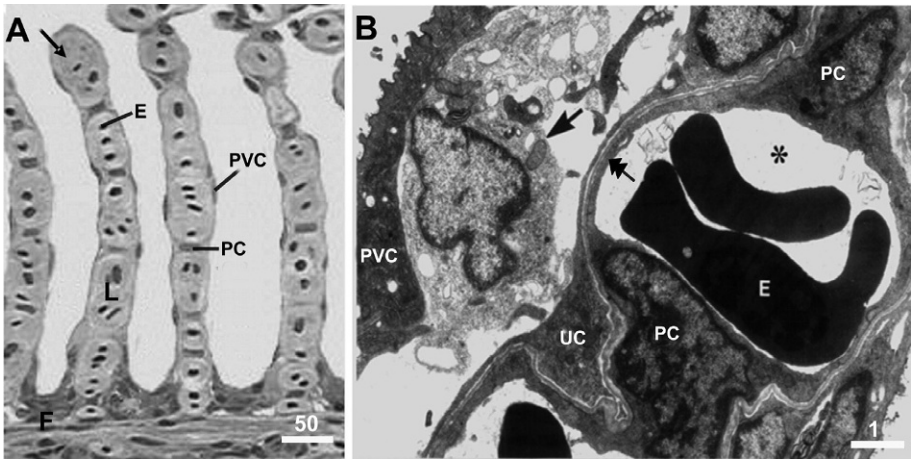

FIG. 10.5 (A) Filament (F) and lamellae (L) of *Prochilodus lineatus* showing the pavement cells of lamellar epithelium, pillar cells (PC), and erythrocytes (E) in the blood spaces. The arrow shows the marginal channel. Scale bar = 50 μm. Photo: A.F. Mazon. (B) Transmission electron photomicrograph of lamella of *Hypostomus plecostomus* showing the pillar cells and blood spaces (*) formed by the pillar cell flanges (double arrow). Note the leukocyte (arrow) between the two cell layers of lamellar epithelium. *PVC*, pavement cell; *PC*, pillar cell; *UC*, undifferentiated cell; *E*, erythrocyte. Scale bar = μm. *(Photo: S.A. Perna.)*

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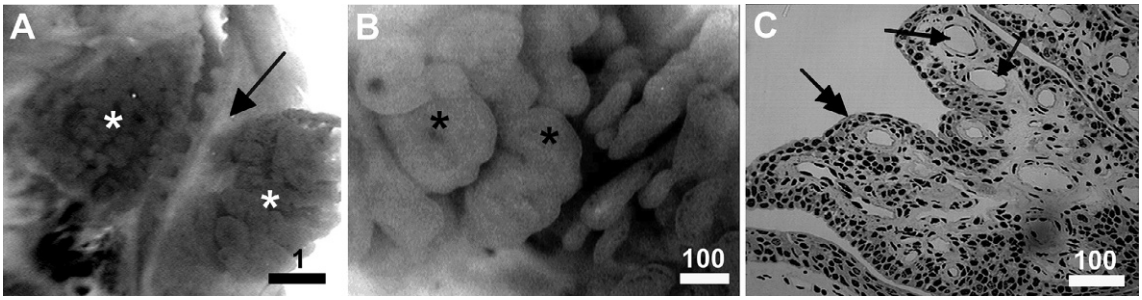

FIG. 10.6 (A–C). *Lepidosirem paradoxa* gills. (A) Short papillary lamellae (*) in the gill arches (arrows). (B) Detail of papillary lamellae (*). (C) Sagittal section of papillary lamellae. Note the absence of pillar cells, the large epithelium thickness (double arrow), and capillaries (arrow). Scale bar = cm in A and μm in B and C. *Photo: M.F.P.G. Moraes.*

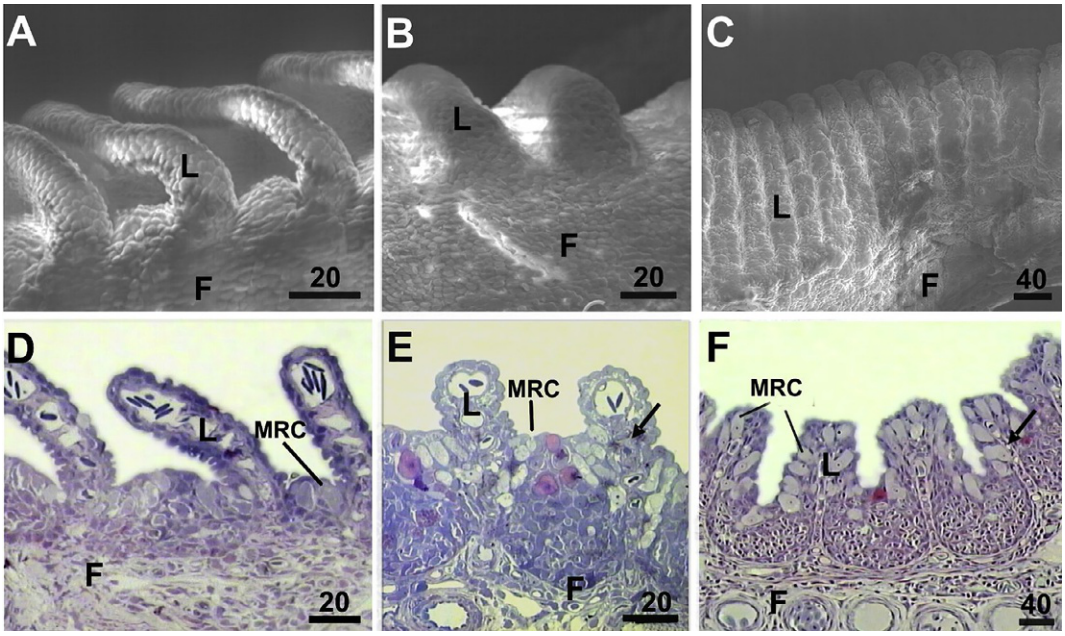

FIG. 10.7 Gills of the lungfish *Arapaima gigas*. (A–C) Scanning electron photomicrograph of filament (F) and lamellae (L) of fish with 105 g (A), 575 g (B), and 1343 g (C). Note the height of lamellae contacting the aquatic environment. (D–F) Longitudinal sections of filament (F) showing the lamellae structure (L) and the thickness of the filament (F) and lamellar (L) epithelia of fish with 105 g (D), 575 g (E), and 1343 g (F). Note the atrophy of pillar cells (arrows) and the mitochondria-rich cells (MRC) in the lamella. Scale bar = μm. *(Photo: C.A. Ramos.)*

lamella to reduce the total surface area, which renders it inefficient for aquatic respiration (Fig. 10.7A–F) (Ramos et al., 2013). Moreover, *A. gigas* uses a modified swim bladder for aerial respiration.

Gill epithelium

The epithelium of the gills is constituted by different cell types: pavement cells (PVC), mucous cells (MC), rodlet cells (RC), undifferentiated cells (IC), neuroepithelial (NC) cells, and mitochondria-rich cells (MRC), also called chloride cells (CC). The filament epithelium is stratified (5–12 layers of cells) depending on the fish behavior or habitat (Mazon et al., 1998; Moron et al., 1996, 2003; Negreiros et al., 2011; Ramos et al., 2013). The outermost cell layer, which is in contact with the aquatic environment, constitutes PVC (>90%) and the innermost cell layer constitutes IC, which are located on the basement membrane, close to the venous sinus. While both the MRC and MC are distributed among PVC (>10%), the RCs are secretory cells and their presence in the filament epithelium is not ubiquitous across species (Leino, 1974; Mendonça et al., 2005). *A. gigas* (Ramos et al., 2013), which are fish of the Curimatidae family, and the erythrinids *H. malabaricus* and *H. unitaeniatus* show numerous RCs in the filament epithelium (Mendonça et al., 2005). Neuroepithelial cells are found in

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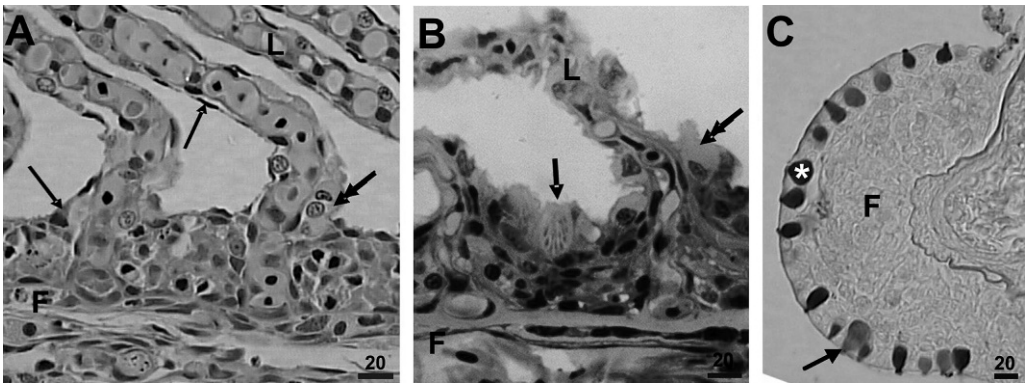

FIG. 10.8 (A) Pavement cell (arrows) and mitochondria-rich cells (double arrow) in the filament (F) and lamellae (L) of *Pimelodus maculatus*. (B) Rodlet cells (arrow) and mitochondria-rich cells (double arrow) in *Hoplias malabaricus*. (C) Mucous cells in *Pimelodus maculatus*: AB-positive cells (*), PAS-positive cels (arrow) (Staining: A and B, Toluidine blue; C, periodic acid of Shiff and Alcian blue pH 2,5). Scale bar in µm. *(Photo: S.E. Moron and M.G. Paulino.)*

the filament epithelium on the efferent side at the intermediate position between the blood and water flow in the lamellae and on the basement membrane near the subepithelial nerve endings. These cells are characterized by granules that presumably contain serotonin that is released in response to environmental hypoxia (Dunel-Erb et al., 1982; Laurent, 1984). Fritsche and Nilsson (1993) demonstrated that NC exhibits structural features like the type 1 cells found in mammalian carotid bodies. These cells were considered possible peripheral O_2 receptors. The lamellar epithelium contains two layers of cells in the basement membrane and forms a thin membrane that separates the external medium from the blood (Fig. 10.5A and B). The inner layer consists of IC; the outer layer is in contact with the aquatic environment and consists mainly of PVC (Fig. 10.5B). The presence of MRC in the lamellar epithelium is related to the water quality of the fish habitat and/or the efficiency of the osmo-ionic regulation of the species (Fig. 10.8A). Unlike the freshwater teleost, in the freshwater ray from the Amazon region, the epithelial cell layers of filament, except those of the layer located near the basement membrane, present positive periodic-acid of Schiff (PAS) and Alcian blue (AB) reaction (Fig. 10.9A and B) (Duncan et al., 2010).

The PVCs are characterized by an electron-dense cytoplasmic matrix, a well-developed Golgi complex, abundant rough endoplasmic reticulum, numerous ribosomes, and few mitochondria (Fig. 10.10A–C). The PVCs of freshwater stingrays have numerous secretory granules positive to PAS and AB, with a pH 2.5 staining immediately below the apical membrane (Duncan et al., 2010). The junctions between PVCs are tight (700 nm), including 1–4 desmosomes; many desmosomes characterize the intercellular junctions between PVC and MRC, especially in the erythrinid *H. malabaricus* (Moron and Fernandes, 1996). The apical surface of PVC is characterized by microvilli or short or long microridges, depending on the species. These surfaces have a polygonal shape with a generally well-defined cell boundary (Fig. 10.10E and F) (Fernandes and Perna-Martins, 2001; Fernandes et al., 2013; Moron and Fernandes, 1996; Paulino et al., 2012). In some species, the PVC of the lamellar epithelium has a smooth surface with a long microridge at the cell limit (Figs. 10.10D and 10.11C–D). Between the filament and lamella close to the lamella basement, some species exhibit a transitional region showing PVC with an apical surface of short and sparse microridges (Fig. 10.10D) (Mazon et al., 2002; Moron and Fernandes, 1996). The PVC in freshwater teleosts may play an important role in ion absorption and acid-base balance (Evans et al., 2005) as in the rainbow trout (*Oncorhynchus mykiss*) and tilapia of Mozambique (*Oreochromis mossambicus*), which present subpopulations of PVC rich in mitochondria and V-ATPase (Galvez et al., 2002; Goss et al., 2001). However, this characteristic was not investigated in the neotropical species.

The MRCs are distributed in the gill epithelium mainly in the interlamellar region and near the base of the lamellae. Depending on the environmental conditions, these cells are found in the lamellar epithelium (Figs. 10.8A–B, 10.9E, 10.11B–D). In the Brazilian freshwater fish species, MRCs are common in the lamellar epithelium because most Brazilian continental waters have low Na^+ and Cl^- concentrations and low hardness (Fernandes et al., 1998; Moron et al., 2003). However, the differences between species living in the same environment are related to the efficiency of osmo-ionic regulation and the mode of respiration. For example, for the erythrinids traira, *H. malabaricus,* and jeju, *H. unitaeniatus*, living in the same environment, the distribution of MRC differs (Fig. 10.11A–D). In *H. malabaricus*, an exclusively aquatic-breathing species, MRCs are rare in the lamellae (Fig. 10.11A), whereas in *H. unitaeniatus*, a facultative air-breathing species, MRCs occupy 50%–60% of the outermost epithelial cell layer of lamella (Fig. 10.11B). In the filament, the apical

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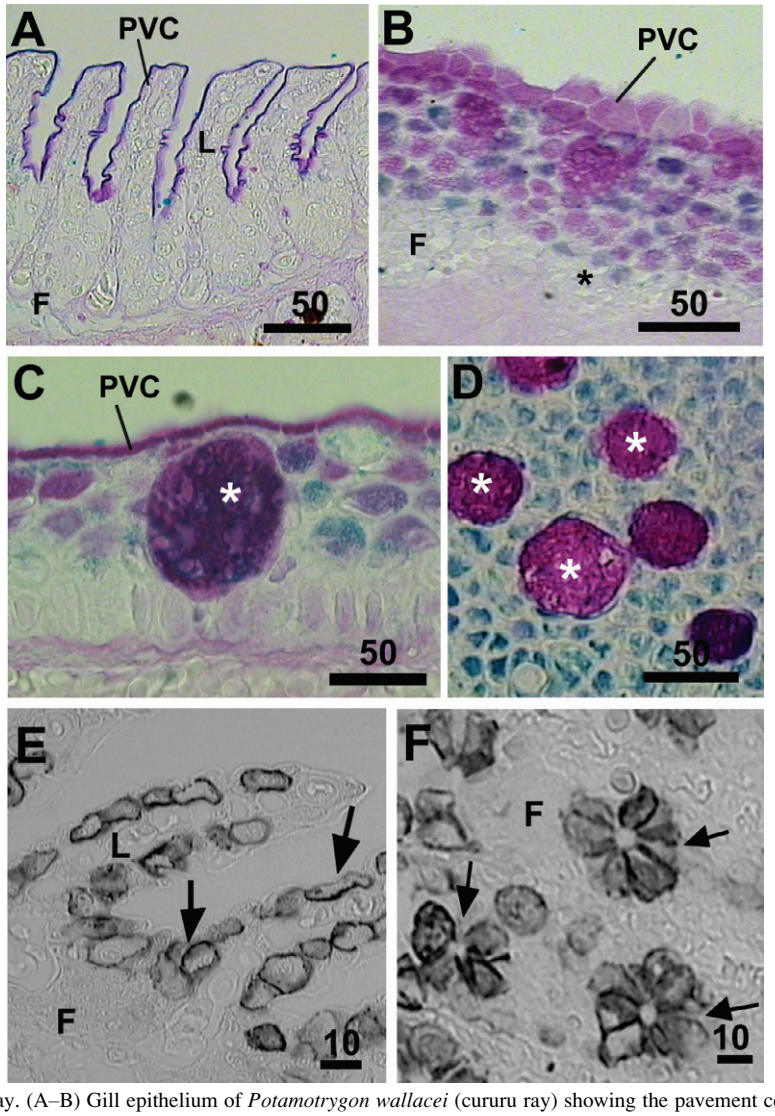

FIG. 10.9 Freshwater stingray. (A–B) Gill epithelium of *Potamotrygon wallacei* (cururu ray) showing the pavement cells in the lamellae in (A) and filament in (B) stained with the periodic acid of Shiff (double arrow) and Alcian blue (arrow). * indicates undifferentiated cells. (C–D) Giant mucous cells (*) in the filament epithelium of *Potamotrygon schroederi*. (E) Mitochondria-rich cells (arrows) in the lamellae of *Potamotrygon schroederi*. (F) Mitochondria-rich cells (arrows) in the filament epithelium of *Potamotrygon wallacei*. Scale bar in μm. *(Photo: W.P. Duncan.)*

area of MRCs is greater in *H. unitaeniatus* (Fig. 10.11C and D). The MRCs are the sites of active absorption of Na^+, Cl^-, and Ca^{2+} ions in freshwater fish.

The MRC has a round nucleus and is characterized by numerous mitochondria and a complex tubular system formed by cellular membrane invaginations in which there are Na^+/K^+-ATPase enzyme units (Hirose et al., 2003; Kaneko et al., 2008; Philpott, 1980; Wilson, 2007). The tubular system is well developed in most neotropical freshwater species such as *H. tietensis*. The apical membrane of the MRC may present microvilli or a spongy-like appearance (Fig. 10.10E–H) (Fernandes et al., 2013; Costa et al., 2017). In the same fish species, it is possible to find populations of MRC presenting both apical surface types. In freshwater elasmobranchs, the MRCs are like those of the teleost and are characterized by an apical surface with numerous microvilli. However, the tubular system is poorly developed, and the $Na^{+/}K^+$-ATPase enzyme units are primarily found in the basolateral membrane folds (Duncan et al., 2010). The stingrays commonly known as the cururu ray, *Potamotrygon wallacei,* (early named *Potamotrygon* sp (Carvalho et al., 2016)), have MRCs distributed individually in the lamellar epithelium and grouped in a follicular arrangement in the filament epithelium (Fig. 10.9F), similar to that found in the cyprinid *Tribolodon hakonensis* that lives in acid waters (pH 3.4–3.8) from Ozorezan Lake, Japan (Hirata et al., 2003). Considering that *P. wallacei* is endemic to Rio Negro in the Amazon region,

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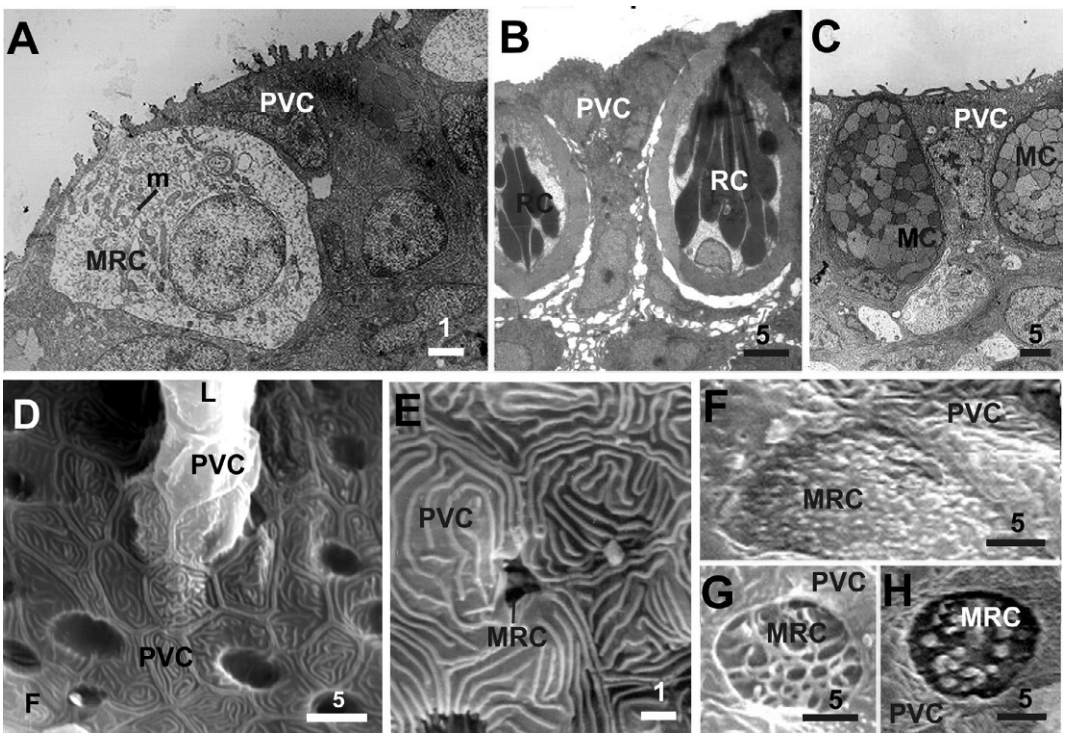

FIG. 10.10 (A–C) Transmission electron photomicrograph of gill epithelium showing the pavement cells (PVC) and mitochondria-rich cells (MRC) of *Hoplias malabaricus* in (A), Rodlet cells (RC) of *Arapaima gigas* in (B), and mucous cells (MC) of *H. malabaricus* in (C). (D–H). Scanning electron photomicrograph of gills showing the surface architecture of PVC in the filament and lamella in (D) and CPV and MRC in the filament of *H. malabaricus* in (E) and MRC in the filament of *Hypostomus tietensis* in (F–H). Note the differences in the apical surface of CRM. *M*, mitochondria. Scale bar in μm. *(Photo: S.E. Moron and C.A. Ramos.)*

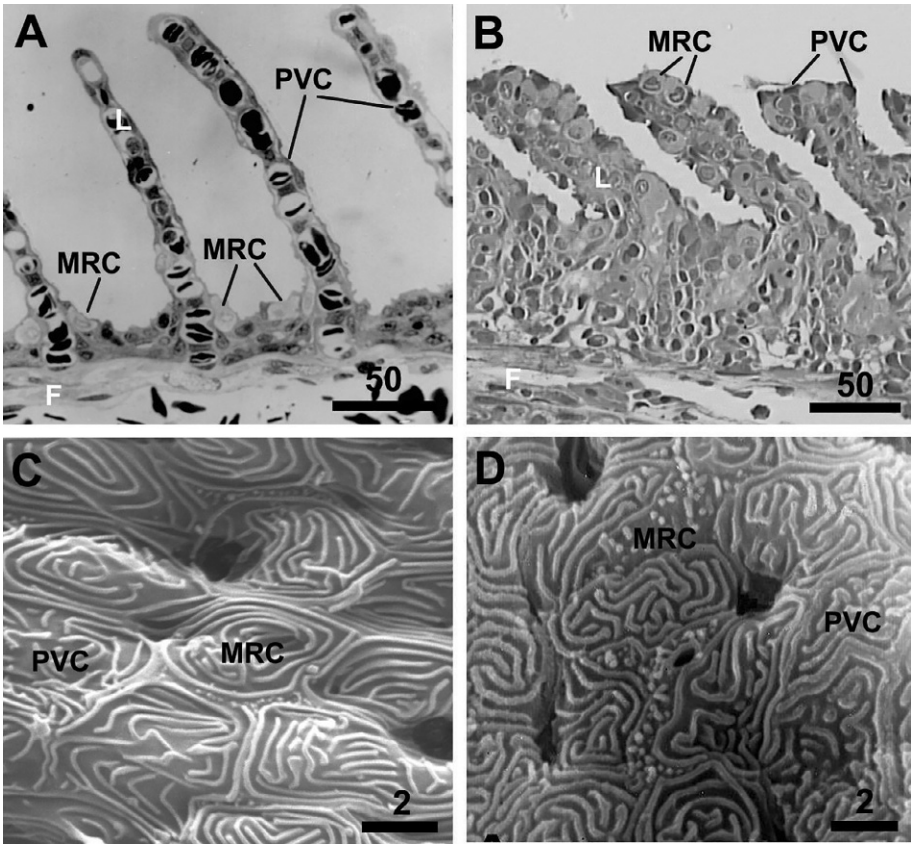

FIG. 10.11 (A and B) Gill filament and lamella of traíra, *Hoplias malabaricus* (A), and jeju, *Hoplerythrinus unitaeniatus* (B). Note the lamellae thickness and the mitochondria-rich cells (MRC) in the lamellae of jeju. (C–D) Scanning electron photomicrograph of filament epithelium of traíra (C) and jeju (D). Note the apical surface of MRC in the filament epithelium of jeju. *PVC*, pavement cells. *(Photo: S.E. Moron.)*

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whose water is acidic and ion-poor, this follicular organization may be an autapomorphy within the Potamotrygonidae family (Duncan et al., 2010; Duncan et al., 2011).

The MCs are distributed mainly on the edges of the filaments on both the afferent (relative to the position of aa1) and efferent (relative to the position of ea1) sides. These cells are rare in the interlamellar region and lamellar epithelium (Figs. 10.8C and 10.9C–D), except in the facultative air-breather *H. unitaeniatus*, which has MCs in the lamellar epithelium as well (Moron et al., 2009). Granules with different electron densities can be identified in MCs under transmission microscopy (Fig. 10.10C). Different types of MCs can be identified in the fish gills (Figs. 10.8C and 10.9C–D). MCs containing neutral mucosubstances (hexoses and sialic acid) present a positive reaction to period acid and Shiff reactive (PAS-positive). MCs containing sulfated mucosubstances (sulfated ester and carboxyl group) present a positive reaction to Alcian blue, pH 2.5 (AB 2.5-positive) whereas those MC containing sulfated acid mucosubstances present a positive reaction to Alcian blue, pH 1.0 (AB 1.0-positive). Some MCs may contain all substances listed above. For instance, *Astyanax fasciatus* (lambari) and *Pimelodus maculatus* (mandi) have five MC types distributed in the filament epithelium (Paulino et al., 2014), *Prochilodus lineatus* (curimbatá) has four types (Paulino et al., 2012), and *A. gigas* has two MC types (Ramos et al., 2018). The giant MC of the freshwater stingrays of the Amazon region are PAS and AB positive (Duncan et al., 2010). A neutral mucosubstance has a low viscosity and lubricates the gill epithelium (Sibbing and Uribe, 1985). Acid and sulfated acid mucosubstances have high viscosities, facilitating the adhesion of suspended particles in the water and protecting against the proliferation of potentially pathogenic microorganisms in the branchial epithelium (Mittal and Mittal, 2008). The thin mucus layer on the epithelial cells protects against mechanical injury and, together with the glycocalyx on the membrane of the PVC, helps the osmotic, ionic, and acid-base regulation mechanisms in the gills. This is because the charges of the radicals on the surface of the mucosubstances and the glycocalyx attract ions, keeping them close to the gill surface and generating an ion gradient that favors ionic regulation. The MCs respond to changes in the ion concentration in the aquatic environment and the presence of xenobiotics via proliferation of a given cell type or modifying the mucosubstances produced by the cells (Moron et al., 2009; Paulino et al., 2012, 2014).

Accessory organs for air breathing

Many fish species have accessory organs for air breathing. Air respiration in fish occurs in approximately 125 genera and is distributed in 49 families, most of which live in tropical and subtropical waters; this mode of respiration usually occurs when fish are exposed to environmental hypoxia/anoxia or in response to increasing the metabolic activity (Graham, 1997). Two categories, amphibian and aquatic air breathers, were defined by Graham (1997) according to behavior and habitat. Amphibian air breathers are those fish that take O_2 from the air when they are temporarily out of the aquatic environment. These species emerge for feeding, spatial orientation, escape from predators, drought, marking territory, and/or cutting behavior for mating. There are two distinguishable fish groups among the amphibian air breathers: the active fish on land that exhibit voluntary emersion and can migrate from one body of water to another, and the inactive fish who can only support brief exposure periods due to the disappearance of their habitat during low tide in marine coastal areas or due to the dry season in the tropical environments (Graham et al., 2007). In the latter case, these fish become confined in mud burrows and enter into an estivating state of metabolic depression. Certain families that enter into this state include Lepidosirenidae and Synbranchidae (Bicudo and Johansen, 1979; Harder et al., 1999; Sawaya, 1946). Aquatic air breathers are those who remain in the water and surface periodically to engulf air; they are divided into facultative and continuous air breathers (Cruz et al., 2013; Jucá-Chagas, 2004; Mattias et al., 1996a). Aquatic facultative air breathers do not breathe atmospheric air in the environmental normoxic conditions (O_2 saturated water), but use air respiration when exposed to unfavorable conditions for aquatic breathing such as hypoxia (reduction of O_2), hypercapnia (increased concentration of CO_2), or an increase in metabolic activity (increased temperature into water or physical activity). Aquatic continuous air breathers take air at regular intervals, even in normoxic water (Cruz et al., 2009; Graham et al., 2007), and may be divided into two types: obligatory and nonobligatory (Graham et al., 2007). The aquatic obligatory air-breather fish (such as *A. gigas*) do not survive in water, even in normoxic conditions, and require O_2 supplementation of the atmospheric air (Brauner et al., 2004; Brauner and Val, 1996; Gonzalez et al., 2010; Hulbert et al., 1978; Sawaya, 1946; Stevens and Holeton, 1978; Val and Almeida-Val, 1995). The aquatic nonobligatory air breather meets the O_2 for its metabolic needs in normoxic water while it takes the atmospheric air only in hypoxic waters (Mattias et al., 1998; Takasusuki et al., 1998).

The diversity of the air-breathing organ structure is related to the selective forces that have affected the evolution and irradiation of fish. For instance, dipnoi (*Neoceratodus, Lepidosiren, Protopterus*) have lungs (Icardo, 2018; Kemp, 1987; Maina and Maloiy, 1985; Moraes et al., 2005) and a number of teleosts such as *Arapaima* (Ramos et al., 2013; Sawaya, 1946; Val, 1996), *Gymnotus* (Liem, 1989; McKenzie et al., 2012), and *Hoplerythrinus* (Fernandes and Moron, 1996; Kramer, 1978; Mattias et al., 1996b; Pelster et al., 2018) use a modified swim bladder for air respiration (Fig. 10.12A

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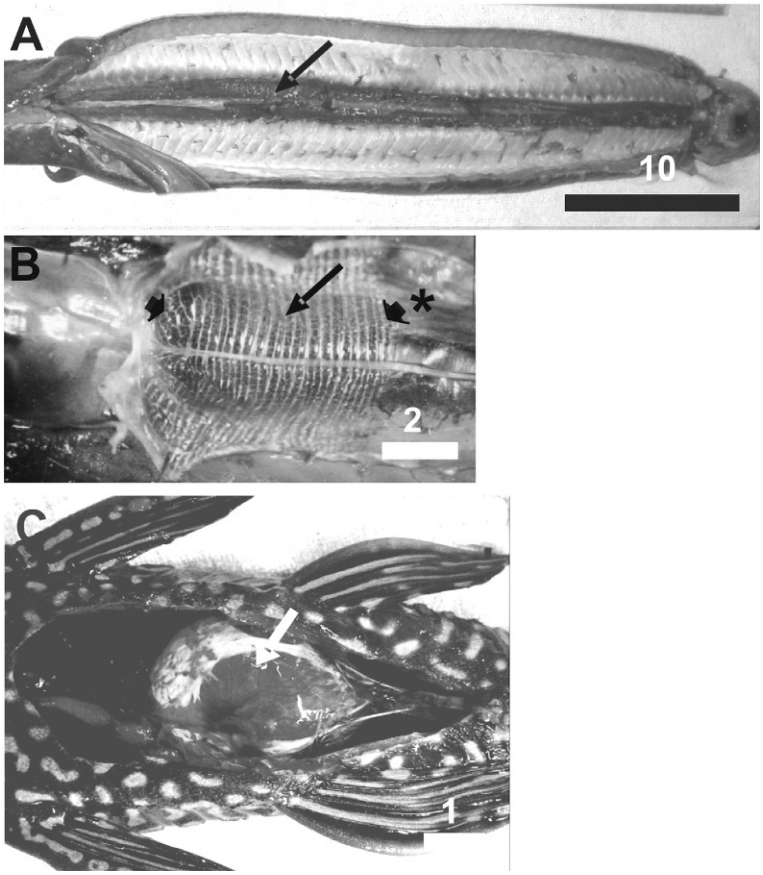

FIG. 10.12 Accessory air-breather organs. (A) Lungs in *Lepidosiren paradoxa* (Photo: M.G. Paulino). (B) Swimming bladder in *Hoplerythinus unitaeniatus* (Photo: S.E. Moron). (C) Stomach in *Pterygoplichthys anisitsi*. *(Photo: A.L. Cruz.)*

and B). The loss of the pneumatic duct during the evolution of the teleost led to the appearance of a great diversity of accessory organs for air breathing (Graham, 1997). In these groups, most of the accessory organs for air breathing are in the head region and consist of the buccal and pharyngeal epithelium, such as those organs in *Electrophorus*, *Monopterus*, *Blennius,* and *Gobios*. These organs include pharyngeal pouches such as in *Channa* and *Monopterus*, and modified branchial, palate, and opercular gills like those in *Clarias* (Munshi, 1985), *Anabas* (Munshi, 1985), and *Gobionellus* (Aguillar et al., 2018). Moreover, *Peryophthalmus* have gills adapted to air breathing in the terrestrial environment (Graham et al., 2007). Adapting to air breathing is also localized in the stomach and gut of some fish, such as in *Hoplosternum* (Gee and Graham, 1978; Jucá-Chagas and Boccardo, 2006), *Hypostomus* (Carter and Beadle, 1931), *Loricariichthys* (Silva et al., 1997), *Ancistrus* (Satora, 1998), *Corydoras* (Podkowa and Goniakowska-Witalinska, 2002), and *Pterygoplichthys* (Cruz et al., 2009) (Fig. 10.12C).

Gill morphometry and accessory organs in relation to respiratory function

Morphometry is relevant in interpreting the function of a given organ. The morphometry of the gills and accessory organs provides information about the O_2 demand, the gas diffusion capacity, and the adaptation to different environments and adverse conditions (Fernandes et al., 2007; Hughes, 1980; Saroglia et al., 2007) as well as interspecific and intraspecific relationships related to respiratory and nonrespiratory functions (Chapman, 2007; Galis and Barel, 1980). Different methodologies have been described from the classical method proposed by Hughes (1966), which is based on gill geometry but does not apply to deeply modified gills (see review: Saroglia et al., 2007). Recently, a new method based on the principles of stereology was described in order to estimate the respiratory surface and morphometric diffusion capacity (Costa et al., 2007). This method can be applied to all types of gills and accessory organs (Aguillar et al., 2018; Cruz and Fernandes, 2016; Cruz et al., 2009, 2013; Moraes et al., 2005).

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TABLE 10.1 Morphometry of gill elements and total and specific respiratory surface of three ecologically distinct species of the family Erythrinidea: *Hoplias malabaricus*, *Hoplias lacerdae*, and *Hoplerythrinus unitaeniatus*.

Gill elements	*Hoplias malabaricus*	*Hoplias lacerdae*	*Hoplerythrinus unitaeniatus*
	(251 g)	(243 g)	(247 g)
Filament			
Number	2904	2860	2484
Mean length (mm)	5.6	3.7	3.5
Total length (mm)	16,269	10,725	8843
Lamella			
Number/mm filament	44	42	42
Total number	793,758	450,450	371,408
Bilateral area (mm^2)	0.11	0.09	0.06
Total respiratory area (mm^2)	87,314	40,541	22,284
Specific respiratory area ($mm^2\ g^{-1}$)	348	169	90

Gill arches have different lengths that decrease in size from the first to fourth branchial arch. Furthermore, the gill filaments inserted in each branchial arch have different lengths along the branchial arch, evidently adapting to the size of the opercular cavity. Differences in the number and length of gill filaments as well as the frequency and dimensions of the lamella determine the respiratory surface area and the resistance to water flow between lamellae and, consequently, the efficiency of gas exchange. Active species, presumably species with a high metabolic demand, tend to have long and thin filaments, greater frequency of relatively small lamellae, large respiratory surface areas, and a short diffusion distance between water and blood. Less active species that inhabit a lentic habitat or have accessory organ for air breathing show a small number of filaments, low frequency of lamellae, and consequently low respiratory surface areas (Crampton et al., 2008; Fernandes and Rantin, 1986a; Fernandes et al., 2007; Hughes, 1970; Perna and Fernandes, 1996; Santos et al., 1994; Severi et al., 1997, 2000). However, morphometric studies have shown that, in tropical and subtropical environments, species with exclusively aquatic respiration and low swimming activity that live in predominantly hypoxic lentic environments may present similar gill characteristics to those of active species with high metabolic demands, as in the case of *H. malabaricus* (Fernandes and Rantin, 1985; Fernandes et al., 1994). Table 10.1 shows the size of the gill structures and the respiratory surface area of three ecologically distinct erythrinids, showing the importance of the filament length, frequency, and individual area of the lamellae on the respiratory surface area. Table 10.2 shows the lamellar frequency per mm filament, the bilateral area of lamella, and the respiratory surface area in species with aquatic and aerial respiration.

The respiratory surface area (S_R) of gills correlates with the body mass (M_B), which can be described as $S_R = aM_B^b$. The gill surface varies between species, and generally the value of b is approximately 0.7–0.8, but reaches a value above 1 as in *H. malabaricus* (Table 10.2). Typically, the value of b in a species with accessory air-breathing capabilities is lower than those with exclusively aquatic respiration (Table 10.2). The rate of growth of the respiratory surface area in relation to body mass is related to taxonomy (Hughes, 1980), O_2 absorption (Hughes, 1984), activity (Mazon et al., 1998), environment (Fernandes et al., 1994), and the degree of dependence of aquatic or aerial environments for respiration in those species that have bimodal or multimodal respiration (Cruz and Fernandes, 2016; Fernandes et al., 1994; Mattias et al., 1996b; Ramos et al., 2013).

The diffusion distance between water-blood or the diffusion barrier of O_2/CO_2 is determinant in the diffusion capacity of the gases. The diffusion capacity (D) of the respiratory gas is expressed in moles of gas per unit time and the difference in the partial pressure of that gas through the membrane separating the respiratory medium (water) from the transport medium (blood). According to Fick's Law, the diffusion capacity can be expressed in two ways:

(1) $D_{morfol} = KS/t$,

where D_{morfol} is the morphological diffusion capacity and represents the maximum diffusion capacity of the organ, K is Krog´s diffusion constant, S is the surface area of the gas exchange organ, and t is the diffusion barrier thickness (the S/t refers to the anatomical diffusion factor, FDA),

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TABLE 10.2 Lamellar frequency/mm of filament (two sides) (L-100 g/mm F), bilateral surface area of lamella (S_L-100 g), and total respiratory surface area (S_R-100 g) for 100-g fish and b coefficient of allometric relationships between respiratory surface area (S_R) and body mass (M_B) given by the equation $S_R = aM_B^b$ of some freshwater fish from neotropical (N) and other world regions.

Species	L-100 g/mm F (n/mm)	S_L-100 g (mm^2)	S_R-100 g (mm^2)	b coefficient	References
Aquatic respiration					
Hoplias malabaricus (N)	46	0.048	24,005	1.14	Fernandes et al. (1994)
Hoplias lacerdae (N)	44	0.063	20,498	0.81	Fernandes et al. (1994)
Hypophthamus edentatus (N)	46	0.028	6639	0.89	Moron et al. (1996)
Piaractus mesopotamicus (N)	69	0.025	19,502	0.77	Severi et al. (1997)
Plagioscion squamosissimus (N)	54	0.063	30,141	0.70	Mazon et al. (1998)
Prochilodus lineatus (N)	49	0.094	44,610	0.72	Mazon et al. (1998)
Tilapia rendalli	41	0.018	7040	1.08	Meletti et al. (1998)
Oreochromis niloticus	42	0.036	17,010	0.75	Fernandes and Rantin (1986a)
Bimodal respiration (aquatic and aerial)					
Anabas testudineus	36	–	4700	0.61	Hughes et al. (1973)
Channa punctata	38	–	7200	0.59	Hakin et al. (1978)
Hoplerythrinus unitaeniatus (N)	46	0.043	12,500	0.67	Fernandes et al. (1994)
Hypostomus regani (N)	38	0.048	7041	0.80	Mattias et al. (1996b)
Hypostomus Plecostomus (N)	48	0.015	9400	0.67	Perna and Fernandes (1996)
Rhinelepis strigosa (N)	26	0.025	20,137	0.76	Santos et al. (1994)

(2) $D_{physol} = M/\Delta P$,

where D_{physol} is the physiological diffusion capacity that depends on several factors and represents the diffusion capacity under a given physiological condition, M is the rate of gas transfer rate O_2/CO_2, and ΔP is the difference of the partial pressure of the gas between the diffusion membrane of the external medium/blood.

Dmorfol emphasizes the importance of respiratory surface area and diffusion barrier. The water-blood diffusion barrier consists of lamellar epithelium, a basement membrane, and the flange of the pillars. It varies between species, being thicker in sluggish fish or those that have bimodal respiration and thinner in active fish or those that are exclusively water-breathing that face hypoxic water in the habitat (Costa et al., 2007, 2017; Cruz and Fernandes, 2016; Cruz et al., 2009; Hughes et al., 1973; Moron et al., 2003; Sakuragui et al., 2003).

Respiration

The gas exchange (O_2/CO_2) in the respiratory organs depends on the ventilation of the respiratory surface and blood circulation in the respiratory units (lamella). In fish gills, gas exchange occurs by diffusion in the countercurrent (water/blood flow), which maintains the O_2 diffusion gradient across the gill epithelium along the entire length of the lamella. The volume and velocity of the water flow on the gills depends on the gill ventilation, that is, water movement between the lamellae.

Gill ventilation consists of four phases and involves a system of pumping water through the gills via a pressure pump located in the buccal cavity and the suction pumps located in the opercular (right and left) cavities that generate a nearly continuous flow of water on the gills (for details, see Moyes and Schulte, 2007). The ventilation in most species is continued as in *H. malabaricus, H. lacerdae* (Rantin et al., 1992, 1993), and *O. niloticus* (Fernandes and Rantin, 1987, 1989).

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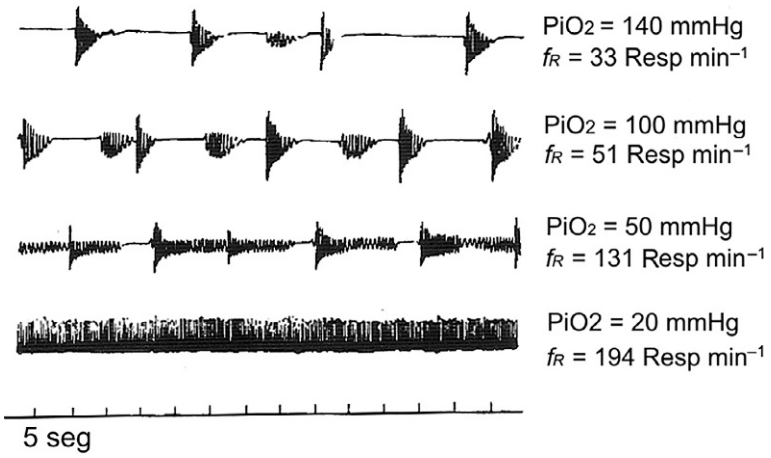

FIG. 10.13 Respiratory cycles (respiratory frequency $= f_R$) in *P. lineatus*, in normoxia ($PiO_2 = 140$ mmHg) and hypoxia ($PiO_2 = 100$–20 mmHg), at 25°C. The respiratory cycles are folowed by long apnea period. In moderate hypoxia ($PiO_2 = 100$ mmHg), the apnea period is reduced and, in severe hypoxia ($PiO_2 = 50$–20 mmHg) the respiratory cycle become continuous. *(Modified from Fernandes, M.N., Barrionuevo, W.R., Rantin, F.T., 1995a. Effect of thermal stress on the respiratory responses to hypoxia of a South America Prochilodontidae fish, Prochilodus scrofa. J. Fish Biol. 46, 123–133.)*

However, some species exhibit periods of rapid ventilatory cycles followed by periods of apnea such as in the *Cyprinus carpio* (carp) (Hughes et al., 1983; Glass et al., 1991; Glass and Soncini, 2007) and in the Neotropical fish *P. lineatus* (Fernandes et al., 1995a) (Fig. 10.13). Very active species use "ram" ventilation, in which these species swim very fast while keeping the mouth open; water that enters the mouth is forced to pass over the gills without buccal and opercular pumping. Many fish use buccal and opercular pumps when at rest or swimming slowly and use "ram" ventilation during high-speed swimming. Among the species of freshwater Brazilian ichthyofauna, there is evidence that the mapará, *Hypophthalmus edentates*, use "ram" ventilation and depends on this type of ventilation to obtain the O_2 necessary for metabolic demand. The species has a reduced branchial pumping apparatus and swims fast and continuously when not confined; if the species is confined, it does not survive, even when in oxygenated water.

During ventilation, the anatomical and physiological dead space (see: Hughes, 1966; Waterman et al., 1971) varies depending on the ventilation volume and breath rate. Anatomical dead space refers to water volume that does not flow between the lamellae and physiological dead space refers to water volume that flows between the lamellae without any gas exchange (Fig. 10.14A–C). Species with high lamellar frequencies and reduced interlamellar space have greater efficiency in gas exchange by having reduced physiological dead space than those with lower lamellar frequency. Very high ventilatory volume results in large opercular expansion and a greater volume of water may therefore flow through the anatomical dead space. This large volume reduces the contact period between the water and the lamellar surface, thus reducing the diffusion rate (Fig. 10.14B and C). Similarly, a very high breath rate increases the water flow velocity between lamellae and has a similar effect on increasing respiratory volume by reducing the rate of gas diffusion between water and blood (Fig. 10.14C). Therefore, until a certain limit, increasing gill ventilation is beneficial for fish to maintain O_2 uptake; however, very high ventilation values increase the anatomical and physiological dead spaces and decrease the effectiveness of gas exchange at the gill surface, reducing the extraction of O_2 by the gills. To minimize this effect, circulatory mechanisms, also up to a certain limit, help maintain the optimal ventilation/perfusion ratio for gas exchange.

The energetic cost of gill ventilation is high because of the viscosity of the water respiratory medium as compared to air and the low O_2 solubility in water. Therefore, an increase in gill ventilation results in the additional energy expenditure for respiration. Few studies have evaluated the metabolic cost of gill ventilation, with values ranging from 2% to 50% depending on the species and the O_2 concentration in the environment (Shelton, 1970). In the neotropical species, the metabolic cost of branchial ventilation ranged from 2% to 5% at rest and reached 13% to 18% in severe hypoxia (Fernandes and Rantin, 1994; Rantin et al., 1992).

Both the blood flow in the filaments and the blood spaces between the pillar cells are associated with cardiovascular events that control blood flow and pressure as the result of cardiac performance and changes in the systemic and branchial circulation. Gill circulation occurs in synchronicity with gill ventilation and the efficiency of the gas exchange depends partly on the ventilation/perfusion ratio. In fish at rest, the lamellar perfusion corresponds to approximately one-third of the lamellae (Farrel et al., 1979). In many lamellae, blood circulation is restricted to proximal and marginal channels. Under conditions of increased physical and/or metabolic activity or reduction of O_2 partial pressure (PO_2) in the aquatic

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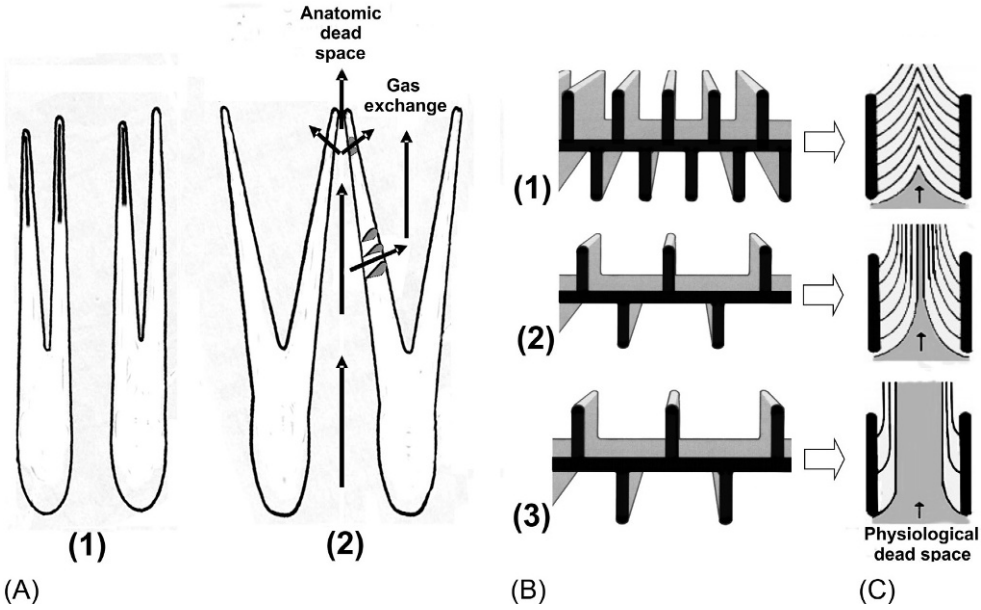

FIG. 10.14 (A) Anatomic dead space. Scheme representing two adjacent gill arches and the filament movement during the gill ventilation: (1) Contracted adductors muscles, (2) Contracted abductor muscles. The free ends of filaments from apposite hemibranchs approximate forcing the water goes between lamellae. The arrows indicate the water flow. (B and C) Physiological dead space. Scheme representing the distances between lamellae (1), (2), and (3) in (B) and water flow between lamellae in (C). The gray color represents the water movement without O_2 diffusion to blood: physiological dead space as a function of lamellar frequency/mm filament. The (C) scheme may also represent the increasing of physiological dead space due to increasing ventilatory volume and/or respiratory frequency resulting in an increased velocity of water flow between lamellae, reducing the O_2 diffusion from water to blood. *(Modified from Hughes, G.M., 1966. The dimensions of fish gills in relation to their function. J. Exp. Biol. 45, 177–195.; Waterman, J.B., Frye, B. E., Johansen, K., Kluge, A.G., Moss, M.L., Noback, R.C., Olsen, I.D., Zug, G.R., 1971, Chordate Structures and Function. New York, Macmillan.)*

environment, gill ventilation as well as lamellar recruitment increased the transfer of O_2 from the water to the blood. The efficiency of O_2 extraction by the gills is measured as the percentage of O_2 removed from the water in relation to the total O_2 concentration dissolved in water.

The "cascade of O_2" depends on the resistance of O_2 to diffusion from the gill membranes to the mitochondria, where it is used to produce energy (Wood and Lenfant, 1979). In environmental normoxia, the amount of O_2 that reaches the tissues meets the demand of the animal; however, modifications in the O_2 diffusion resistance can cause internal hypoxia. On the other hand, the reduction of O_2 in the environment (environmental hypoxia) may result in a decrease in O_2 uptake at the gill surface and decreasing PO_2 along the O_2 cascade, also causing internal hypoxia. In order to keep the O_2 cascade unchanged against the O_2 reductions in the aquatic environment, fish use homeostatic cardiorespiratory mechanisms, which maintain the transfer of O_2 from the water medium to the tissues within certain limits. The survival of fish under environmental hypoxia depends on the capability of the animal to detect environmental changes and the efficiency of the compensatory cardiorespiratory mechanisms to maintain the O_2 uptake by the gills.

Oxygen consumption, metabolic rate, and respiratory variables

The O_2 consumption refers to the total consumption of O_2 over a given time. The metabolic rate refers to the consumption of O_2 for a specified time and body mass; both variables can be expressed in moles of O_2 (MO_2) or volume of O_2 (VO_2):

$$\text{Consumption of } O_2 = MO_2\,h^{-1} \text{ or } VO_2\,h^{-1}$$

$$\text{Metabolic rate} = MO_2\,kg^{-1}\,h^{-1} \text{ or } VO_2\,kg^{-1}\,h^{-1}$$

However, many authors use the term O_2 consumption for the metabolic rate, indicating only the unit used to express it.

According to Dejours (1981) and Itazawa and Takeda (1978), VO_2 is calculated by the following equations:

$VO_2 = V_G\alpha_{wO2}\,(PiO_2 - PeO_2)$, where V_G is the gill ventilation, α_{wO2} is the solubility of O_2 in water, PiO_2 is the PO_2 of the inspired water, and PeO_2 is the PO_2 of expired water;

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$VO_2 = (KA/t)\ (PwO_2 - PbO_2)$, where K is the Krogh constant, A/t is the diffusion capacity of O_2 through the gills, PwO_2 is the PO_2 of water, and PbO_2 is PO_2 of blood;
$VO_2 = (Q\beta bO_2)\ (PaO_2 - PvO_2)$ where Q is the blood flow in the gills, βbO_2 is the solubility of O_2 in the blood, PaO_2 is the PO_2 of the arterial blood, and PvO_2 is the PO_2 of the venous blood in the gills.

Both O_2 consumption and the metabolic rate depend mainly on the activity and temperature of the animal and are thus related to body mass. VO_2 increases with body mass (M_B) and can be described by the allometric equation, $VO_2 = aM_B^b$. Changes in environmental O_2 result in changes in the following ventilatory variables: gill ventilation ($V_G = mL_{H2O}\ kg^{-1}\ min^{-1}$), which depends on changes in respiratory volume ($V_R = mL_{H2O}\ h^{-1}\ breath^{-1}$) and frequency ($f_R = breath\ min^{-1}$). The changes in V_G alter the conductance of O_2 by convection and maintain O_2 extraction (%). However, the efficiency of O_2 extraction is due to the ventilation/perfusion ratio. Cardiorespiratory synchronism favors gas exchange; the pulsatile blood flow generated by cardiac contraction is synchronized with the respiratory cycle (buccal and opercular pumps) to maintain a constant pulsating flow of water during the respiratory cycle in order to optimize O_2 uptake.

Ventilatory parameters and O_2 consumption have been addressed using different respirometry techniques. The available respirometry methods for water and bimodal fish face a challenge, as they have been recently reviewed and discussed by several authors in order to measure the physiological performance of organisms (Lefevre et al., 2016; Norin and Clark, 2016; Rodgers et al., 2016; Svendsen et al., 2016a, b)

In fish, rest or routine VO_2 represents the metabolic demand to maintain vital activities at minimum energy consumption in the water column while maximal VO_2 (critical swimming speed, U_{crit}) represents the maximal oxidative metabolic demand of an active animal (see: Davis, 1975) and depends on the cardiorespiratory mechanisms that increase the O_2 capacity of transfer from the water to the blood. Activity above the maximal VO_2 implies the use of an anaerobic metabolism. The scope between the minimum and maximum VO_2 depends on the respiratory surface. The ventilation/perfusion ratio can increase O_2 extraction, which depends on the biochemical characteristics of the blood (Fraser et al., 2006; Val, 1996). In Neotropical air-breathing fish, the contribution of air breathing to aerobic metabolic scope and exercise performance was investigated in the facultative air-breathing knifefish (*Gymnotus carapo*) at 30°C (McKenzie et al., 2012). Under normoxia, the *U*crit and the active metabolic rate with and without access to atmospheric air were similar. Fish having access to air increased the air-breathing frequency with increased swimming speed; 39% of the metabolic rate was derived from air-breathing. However, if access to air was denied, the metabolic rate depended only on gill ventilation, which indicates that this species has facultative dependence of air breathing. Under hypoxic water, the *U*crit and metabolic rate were like normoxia, but 55% of the metabolic rate was derived from air breathing; without access to air, *U*crit and the metabolic rate decreased sharply (McKenzie et al., 2012).

Respiration and environment

Hyperoxia

Environmental hyperoxia may occur during the summer on sunny days as a result of photosynthesis of aquatic plants. In general, water hyperoxia is associated with a low CO_2 partial pressure (PCO_2) and high pH. Due to the high PO_2 in the water, the ventilatory responses decrease and may decrease the lamellar perfusion due to cardiovascular adjustments. This process results in an increase in arterial PCO_2 and a decrease in pH (hypercapnic acidosis) (Wood and Jackson, 1980). In trout, the compensation of blood acidosis occurs in approximately four days due to the accumulation of HCO_3^- in plasma and increasing H^+ secretion through the gill epithelium (Wood, 1991). Morphological responses include a decrease in the height of lamella and an increase of the individual apical surface of MRCs and the fractional area of CCs on the epithelial surface of the gills (Laurent and Perry, 1991).

Hypoxia

Environmental hypoxia is a common occurrence in aquatic environments; for fish, the availability of dissolved O_2 may act as a barrier to species irradiation, depending on their tolerance to environmental O_2 reduction (Chapman, 2007). To a certain extent, permanent or chronic hypoxia in tropical and subtropical regions explains the adaptations that occur to obtain the O_2 needs for metabolism at the behavioral as well as biochemical, physiological, and morphological levels, including the bimodal respiration.

When fish are exposed to hypoxia, their first response is characterized by a behavior to escape. However, the escape response is often not possible (for example, in a hypoxic lake). Then, physiological mechanisms are triggered to

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compensate for the decrease in dissolved O_2. Aquatic surface respiration (ASR) and air breathing are behavioral responses that allow the animal to obtain the O_2 required for the oxidative metabolism; air breathing is therefore a sophisticated form of escape, according to Jensen et al. (1993).

The ASR response allows the fish to obtain the O_2 dissolved in the surface water, which is the more oxygenated layer of the aquatic environment and is a common response in fish of neotropical environments (Kramer and McClure, 1982; Kramer and Mehegan, 1981; Val, 1996; Val and Almeida-Val, 1995). Under hypoxic conditions, the animal swims to the aquatic surface and ventilates the gills. In *Piaractus mesopotamicus* (tambaqui) and *Colossoma macropomum* (pacu), ASR begins upon moderate to severe hypoxia ($PO_2 \sim 70$ and 50 mmHg, respectively), and the fish develops an edema in the lower lip that helps direct water to the oral cavity and gills (Florindo et al., 2006; Rantin and Kalinin, 1996; Rantin et al., 1998). Similar responses were described by Saint-Paul and Soares (1988) for other serrasalmid species such as *Mylossoma duriventris* (pacumirim) and *Mylossoma aureum* (pacu prata). The frequency of ASR increases as the water PO_2 decreases, so the animal can remain on the aquatic surface up to 95% of the time. In these periods, there are cardiorespiratory adjustments evidenced by the increase in f_R and the decrease in heart frequency (f_H) (Rantin and Kalinin, 1996; Rantin et al., 1998). The ASR response implies an increase in the metabolic cost for respiration and the risk of predation by aerial animals (Kramer, 1983).

In general, organs for air breathing are more efficient in obtaining O_2 than excreting CO_2. In *Electrophorus*, O_2 is absorbed by the buccal epithelium and diverticulum and 81% of CO_2 is excreted through the skin (Farber and Rahn, 1970). *Arapaima* depends on the atmospheric O_2 from the ninth day after hatching and gradually becomes exclusively an air-breathing fish (Brauner et al., 2004). Without access to atmospheric air, a 10 g fish can survive two times higher in water than a 1 kg fish (Brauner et al., 2004); approximately 75% of the O_2 is taken up by the swim bladder and 70%–79% of the CO_2 is excreted by the gills (Brauner and Val, 1996; Gonzalez et al., 2010; Hulbert et al., 1978; Randall et al., 1978; Stevens and Holeton, 1978).

Air-breathing fish may survive for a long time in hypoxia and/or anoxia by exclusively using this mode of respiration (Abe, 1995; Abe and Steffensen, 1996a, b; Graham, 1997). The air-breathing frequency in *L. paradoxa*, for example, goes from 3 to 20 breaths h^{-1}, depending on temperature (Johansen and Lenfant, 1967; Jucá-Chagas, 2004; Sawaya, 1946) and has approximately 0.5 mL_{O2} $resp^{-1}$ (Jucá-Chagas, 2004). Lungfish displayed a regular episodic air-breathing pattern at about 1.7 breaths h^{-1} with a mean duration of the air breathing cycle of 10.7 s; the respiratory volume of each cycle is approximately 25.3 mL kg^{-1} (Monteiro et al., 2018).

Facultative air-breather species exhibit different degrees of dependence on atmospheric air, which is directly related to the efficiency of the O_2 uptake from water by the gills. In general, facultative air-breathing fish begin aerial respiration at moderate and severe water environmental hypoxia (Mattias et al., 1996a; Mattias et al., 1998; Nelson et al., 2007; Oliveira et al., 2004; Pelster et al., 2018; Takasusuki et al., 1997, 1998). The air-breathing cycle differs between species. In the Callichthyidae and Cobitidae families, the elimination of respiratory gases occurs via the anus, characterizing a unidirectional flow of gases through the digestive tract; in species of the Loricariidae and Erythrinidae families, CO_2 expiration occurs through the opercular openings (Cruz et al., 2013; Jucá-Chagas, 2004; Jucá-Chagas and Boccardo, 2006; McKenzie et al., 2007; Takasusuki et al., 1997).

The tolerance of exclusively water-breathing fish species depends on physiological and biochemical mechanisms that, to some extent, enable survival in hypoxic environments. The decreasing of water PO_2 can be compensated by reducing the O_2 demand and/or increasing the conductance of the respiratory components. The decreasing of VO_2 during hypoxia has been observed in many fish. In environments exhibiting thermal stratification, fish seek out a region with lower temperature. However, in shallow waters, where the stratification does not occur, the reduction of metabolic demand can be achieved by decreasing motor activity (Wood, 1991). Nevertheless, the escape reaction as well as feeding and reproductive activities are essential in the survival and perpetuation of the species. Therefore, acclimatization to long-term hypoxia implies an increased efficiency in the transfer and transport of O_2 from the environment to the cells. It involves biochemical, physiological, and morphological components such as the expression of hemoglobin isoforms with higher HB/O_2 affinity (Jensen et al., 1993; Rantin and Johansen, 1984; Silva et al., 2014). The acclimatization to hypoxia allows the animal to endure longer periods of environmental hypoxia. However, a reduction of growth rate and reproductive success may result because the energy expenditure or metabolic cost to maintain oxidative demand, even at reduced activity, may affect growth, fertility, fecundity, and the success of offspring (Fernandes and Rantin, 1994; Rantin et al., 1992).

An increase in gill ventilation is the first response to hypoxia; the increasing O_2 conductance by convection implies a greater volume of water passing through between the lamellae per unit time. In other words, V_G is mediated by increasing V_R and f_R. During hypoxia, lamellar recruitment (lamellar perfusion) occurs, which increases the lamella height exposed to the aquatic environment (thus increasing the lamellar area) and reduces the morphological blood-water barrier due to the

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cardiovascular adjustments. This morphofunctional change has been considered an important event for compensating the decrease in the O_2 diffusion gradient from water to blood, according to Fick's Law.

Fish are classified into two groups, oxyconformist and oxyregulator, depending on their ability to regulate VO_2 during gradual hypoxia (Davis, 1975). Oxyconformist species are those that cannot regulate VO_2 during hypoxia and the O_2 consumption decreases following the reduction of environmental PO_2. Most teleosts are oxyregulators and present VO_2 independent of environmental PO_2 until a given PO_2 denominated critical pressure of O_2 (PcO_2). In water where PO_2 is below the PcO_2, VO_2 is dependent on environmental PO_2 and the animal behaves as an oxyconformist. In terms of activity, PcO_2 is defined as limiting incipient oxygen pressure that divides the VO_2 curve as a function of water PO_2 in a respiratory independence zone, whose VO_2 remains constant; the activity in this zone of dependence depends on the environmental PO_2. At rest, PcO_2 is defined as the incipient lethal oxygen pressure and divides the VO_2 curve into a tolerance zone, where VO_2 remains approximately constant with the reduction of the environmental PO_2, and a resistance zone, where VO_2 is dependent on the water PO_2. The resistance zone involves the use of an anaerobic metabolism to complement the energy needs for metabolic activities and the animal survives for a certain time. PcO_2 varies among species and depends on both acclimation temperature and thermal stress and, in general, is lower in tropical and neotropical species (Table 10.3). In some facultative air-breathing species, PcO_2 is lower than water PO_2, which is when aerial respiration begins (Mattias et al., 1996a, 1998; Oliveira et al., 2004; Takasusuki et al., 1998).

TABLE 10.3 Critical oxygen pressure (PcO_2) in different temperatures (Temp.) and body mass (M_B) of neotropical (N) and other freshwater fish with aquatic and bimodal mode of respiration without access to atmospheric air.

Species	M_B (g)	Temp. (°C)	PcO_2 (mmHg)	References
Aquatic respiration				
Brycon amazonicus (N)	155 ± 8	24	25	Monteiro et al. (2013)
Cyprinus carpio	1447–1659	5	11	Ott et al. (1980)
Cyprinus carpio	1447–1659	15	21	Ott et al. (1980)
Cyprinus carpio	1447–1659	25	20	Ott et al. (1980)
Geophagus brasiliensis (N)	220–350	25	18	Fernandes and Rantin (1987)
Hoplias lacerdae (N)	300–500	25	35	Rantin et al. (1992)
Hoplias malabaricus (N)	300–500	25	20	Rantin et al. (1992)
Hoplias malabaricus (N)	24–64	25	27	Kalinin et al. (1993)
Hoplias malabaricus (N)	271–414	25	18	Kalinin et al. (1993)
Hoplias malabaricus (N)	32–115	25	30	Mattias et al. (1996a)
Hoplias malabaricus (N)	265–565	25	21	Sakuragui et al. (2003)
Hoplias malabaricus (N)	246 ± 7	24	21	Monteiro et al. (2013)
Oreochromis niloticus	220–350	25	18	Fernandes and Rantin (1987)
Oreochromis niloticus	232–310	20	19	Fernandes and Rantin (1989)
Oreochromis niloticus	232–310	25	18	Fernandes and Rantin (1989)
Oreochromis niloticus	232–310	30	30	Fernandes and Rantin (1989)
Oreochromis niloticus	232–310	35	30	Fernandes and Rantin (1989)
Piaractus mesopotamicus (N)	558 ± 243	25	34	Rantin et al. (1998)
Prochilodus lineatus (N)	284 ± 11	25	23	Fernandes et al. (1995a)
Prochilodus lineatus (N)	237 ± 11	15[a]	28	Fernandes et al. (1995a)
Prochilodus lineatus (N)	243 ± 19	20[a]	22	Fernandes et al. (1995a)

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TABLE 10.3 Critical oxygen pressure (PcO_2) in different temperatures (Temp.) and body mass (M_B) of neotropical (N) and other freshwater fish with aquatic and bimodal mode of respiration without access to atmospheric air.—cont'd

Species	M_B (g)	Temp. (°C)	PcO_2 (mmHg)	References
Prochilodus lineatus (N)	303 ± 21	30[a]	24	Fernandes et al. (1995a)
Prochilodus lineatus (N)	295 ± 49	35[a]	45	Fernandes et al. (1995a)
Salminus maxillosus (N)	2700–4000	25	42	Souza et al. (2001)
Bimodal respiration (aquatic and aerial)				
Ancistrus chagresi (N)	3–99	25	40	Graham (1983)
Clarias gariepinus	300–700	25	60	Belão et al. (2011, 2015)
Hoplerythrinus unitaeniatus (N)	31–97	25	80	Mattias et al. (1996a)
Hoplerythrinus unitaeniatus (N)	209 ± 5	15	28	Oliveira et al. (2004)
Hoplosternum littorale (N)	44–141	25	57	Affonso and Rantin (2005)
Hypostomus plecostomus (N)	17–85	25	33	Perna and Fernandes (1996)
Hypostomus regani (N)	180–708	25	31	Mattias et al. (1998)
Hypostomus regani (N)	300–600	20	33	Fernandes et al. (1999)
Hypostomus regani (N)	300–600	25	27	Fernandes et al. (1999)
Hypostomus regani (N)	300–600	30	27	Fernandes et al. (1999)
Hypostomus regani (N)	300–600	20[a]	40	Nelson et al. (2007)
Hypostomus regani (N)	300–600	30[a]	82	Nelson et al. (2007)
Pterygoplichthys anisitsi (N)	88–600	25	47	Cruz et al. (2013)
Rhinelapis strigosa (N)	370–845	25	20	Takasusuki et al. (1997)
Synbrachus marmoratus (N)	–	–	40	Bicudo and Johansen (1979)

Values were estimated from the cited references.
[a]*Fish acclimated at 25oC and transferred to the indicated temperature.*

The maintenance of VO_2 is directly associated with ventilatory, cardiac, and blood adjustments to maintain the extraction and transport capacity of O_2. The increasing V_G is the first response to hypoxia and is due to increases in V_R and f_R (Fig. 10.15). At rest, fish have low f_R rates in normoxic or hyperoxic waters, exhibiting three different patterns of breathing: (a) regular continuous breathing as in *H. malabaricus* (Rantin et al., 1992), (b) frequency cycling (continuous breathing with alternating periods of fast and slow breathing varying the amplitude cycling in some instances) as in *Cyprinus carpio* (Glass et al., 1991), and (c) episodic breathing with periods of ventilation separated by periods of apnea as in *P. lineatus* (Fig. 10.13) (Fernandes et al., 1995a, b). Continuous regular breathing is the predominant breathing pattern in fish, but the frequency and amplitude increases under hypoxia. Those species with the breathing pattern b and c increase f_R, changing from episodic to continuous breathing and increasing opercular amplitude.

Among the teleost species more tolerant to hypoxia, the increasing V_G occurs mainly due to a higher increase in V_R than in f_R. According to Rantin et al. (1992), a greater increase in V_R results in a lower metabolic cost during the ventilatory adjustment. Changes in V_G are usually accompanied by a reflex bradycardia (decreasing heart rate), which is compensated by increasing systolic volume and blood pressure; these processes consequently increase the lamellae perfusion (Fritsche and Nilsson, 1993; Rantin et al., 1992). Concomitant cardiac and ventilatory adjustments result in increasing blood volume in the lamellae at each heartbeat, and for a longer time so that improving the oxygen transfer from water to blood occurs. This mechanism maintains the efficiency of O_2 extraction in moderate to severe hypoxia. However, in extremely hypoxic conditions, when the animal is exposed to the environmental PO_2 that constitutes the resistance zone, this mechanism does

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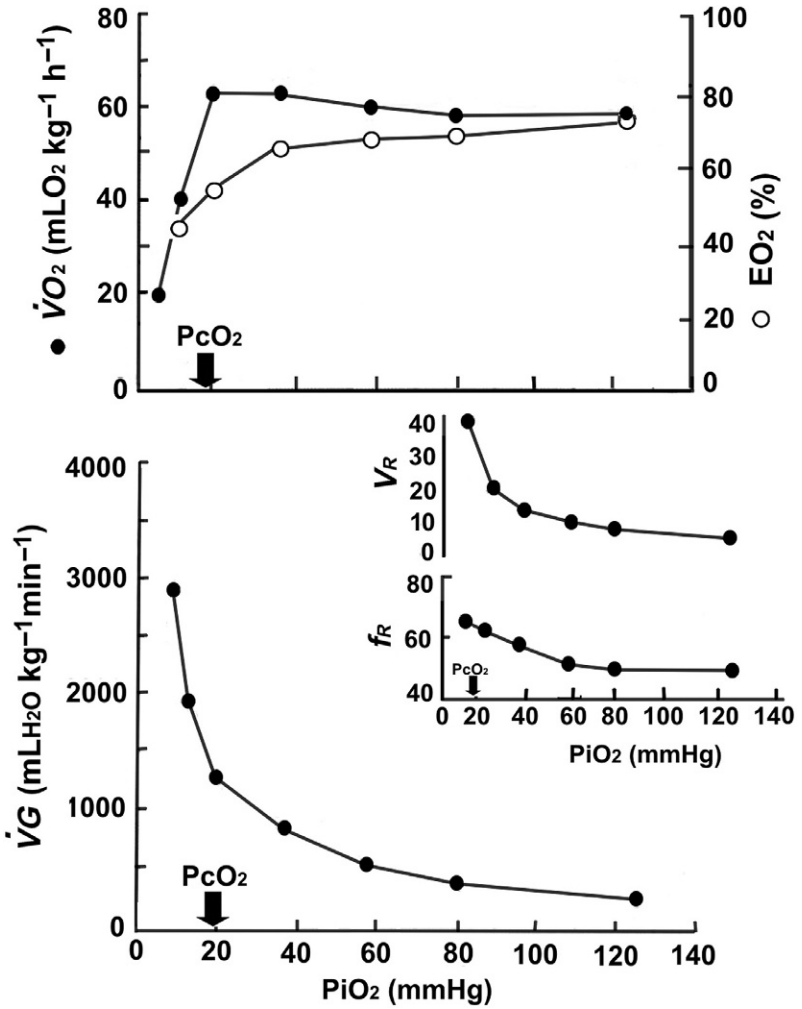

FIG. 10.15 Oxygen consumption (VO_2), O_2 extraction (EO_2), gill ventilation (V_G), respiratory volume (V_R—mL_{H2O} kg^{-1} $resp^{-1}$), and respiratory frequency (f_R—resp. min^{-1}) in relation to a reduction to O2 in water (PiO_2–inspired water) in Nilo tilapia, *Oreochromis niloticus*. *(Data compilation from Fernandes, M.N., Rantin, F.T., 1989. Respiratory responses of Oreochromis niloticus (Pisces, Cichlidae) to environmental hypoxia, under different thermal conditions. J. Fish Biol. 35, 509–519., Fernandes, M.N., Rantin, F.T., 1994. Relationships between oxygen availability and metabolic cost of breathing in Nile tilapia (Oreochromis niloticus): aquaculture consequences. Aquaculture 127, 339–346.)*

not maintain the O_2 extraction due to an increasing anatomical and physiological "dead space." Table 10.4 shows VO_2 in normoxia and hypoxia ($PO_2 = PcO_2$), ventilatory variables, and O_2 extraction in some neotropical and other species.

Facultative air breathing species, without access to atmospheric air, present similar respiratory responses of fish with exclusively aquatic breathing. In *H. regani*, air breathing begins at water $PO_2 = \sim 60$ mmHg, which is 20–30 mmHg above the PcO_2 (Fernandes et al., 1999; Mattias et al., 1998; Nelson et al., 2007). In *H. unitaeniatus*, the PO_2 for which the air breathing begins is size dependent. Smaller species (31–100 g) begin air breathing at water PO_2 levels ranging from 80 to 60 mmHg; the lowest PO_2 corresponds to the PcO_2 when fish do not have access to atmospheric air (Mattias et al., 1996a) while larger specimens begin to breathe air at water PO_2 levels of 44 mmHg, which is approximately the PcO_2 level (40 mmHg) for this size animal (Oliveira et al., 2004). Some species exhibited continuous air breathing. While *H. unitaeniatus* obtained less than 5% of total VO_2 from air breathing in normoxia (26°C), a sharp reduction of water PO_2 (severe hypoxia) increases the air-breathing frequency 10-fold and the VO_2 is maintained unchanged from normoxia (McKenzie et al., 2007). Regardless of animal size, the air-breathing frequency increases with a reduction of environmental PO_2 and without access to atmospheric air, V_G, V_R, and f_R increase continuously until $PO_2 = 30$–20 mmHg; this process is followed by a sharp decrease in the ventilatory parameters at lower water PO_2 (Mattias et al., 1996a; Oliveira et al., 2004).

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TABLE 10.4 Consumption of O_2 (VO_2), gill ventilation (VG), respiratory frequency (f_R), and O_2 extraction (EO_2) of neotropical (N) and other fish in normoxia (N, PO_2 ~140 mmHg) and severe hypoxia (H, PO_2 ~20–30 mmHg) with aquatic and bimodal mode of respiration without access to atmospheric air.

Species	M_B (g)	Temp. (°C)		VO_2 (mLO_2 kg^{-1} h^{-1})	V_G (mL_{H2O} kg^{-1} min^{-1})	f_R (breath min^{-1})	EO_2 (%)	References
Aquatic respiration								
Brycon amazonicus (N)	155 ± 8	24	N	103	746	85	55	Monteiro et al. (2013)
			H	40	743 1	140	31	
Hoplias lacerdae (N)	300–500	25	N	55	250	32	78	Rantin et al. (1992)
			H	60	1400	68	52	
Hoplias malabaricus (N)	300–500	25	N	35	200	42	84	Rantin et al. (1992)
			H	40	1100	64	76	
Hoplias malabaricus (N)	24–64	25	N	38	200	40	–	Kalinin et al. (1993)
			H	40	1500	84	–	
Hoplias malabaricus (N)	271–414	25	N	110	200	42	–	Kalinin et al. (1993)
			H	110	1200	76	–	
Hoplias malabaricus (N)	32–115	25	N	80	210	–	74	Mattias et al. (1996a)
			H	68	3000	–	28	
Hoplias malabaricus (N)	265–565	25	N	42	220	48	–	Sakuragui et al. (2003)
			H	40	2100	75	–	
Hoplias malabaricus (N)	246 ± 7	24	N	64	238	42	85	Monteiro et al. (2013)
			H	40	2625	78	40	
Oreochromis niloticus	220–350	25	N	56	166	38	82	Fernandes and Rantin (1987)
			H	60	1292	55	56	
Oreochromis niloticus	232–310	20	N	39	100	38	85	Fernandes and Rantin (1989)
			H	36	800	50	53	
Oreochromis niloticus	232–310	25	N	59	200	52	80	Fernandes and Rantin (1989)
			H	60	2700	60	48	
Oreochromis niloticus	232–310	30	N	66	270	55	75	Fernandes and Rantin (1989)
			H	60	3500	68	40	
Oreochromis niloticus	232–310	35	N	82	400	58	75	Fernandes and Rantin (1989)
			H	87	–	82	35	
Piaractus mesopotamicus (N)	558 ± 243	25	N	75	500	100	–	Rantin et al. (1998)
			H	38	2500	150	–	
Prochilodus lineatus (N)	284 ± 11	25	N	80	451	57	65	Fernandes et al. (1995a)
			H	90	4500	150	40	

Continued

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TABLE 10.4 Consumption of O_2 (VO_2), gill ventilation (VG), respiratory frequency (f_R), and O_2 extraction (EO_2) of neotropical (N) and other fish in normoxia (N, PO_2 ~140 mmHg) and severe hypoxia (H, PO_2 ~20–30 mmHg) with aquatic and bimodal mode of respiration without access to atmospheric air.—cont'd

Species	M_B (g)	Temp. (°C)		VO_2 (mLO_2 kg^{-1} h^{-1})	V_G (mL_{H2O} kg^{-1} min^{-1})	f_R (breath min^{-1})	EO_2 (%)	References
Prochilodus lineatus (N)	237 ± 11	15	N	52	174	63	71	Fernandes et al. (1995a)
			H	58	1200	100	50	
Prochilodus lineatus (N)	243 ± 19	20	N	54	252	92	61	Fernandes et al. (1995a)
			H	50	1800	165	50	
Prochilodus lineatus (N)	303 ± 21	30	N	129	822	104	60	Fernandes et al. (1995a)
			H	100	3900	150	40	
Prochilodus lineatus (N)	295 ± 49	35	N	184	1217	196	58	Fernandes et al. (1995a)
			H	60	4800	220	30	
Salminus maxillosus (N)	2700–4000	25	N	75	1000	65	–	Souza et al. (2001)
			H	30	3700	80	–	
Bimodal respiration (aquatic and aerial)								
Clarias gariepinus	300–700	25	N	76	769	39	49	Belão et al. (2011)
			H	26	1546	57	20	
Hoplerythrinus unitaeniatus (N)	31–97	25	N	80	1000	–	22	Mattias et al. (1996a)
			H	25	2800	–	20	
Hoplerythrinus unitaeniatus (N)	250 ± 50	25	N	100	500	60	70	Oliveira et al. (2004)
			H	60	4500	80	25	
Hoplosternum littorale (N)	44–141	28	N	75	–	125	–	Affonso and Rantin (2005)
			H	40	–	170	–	
Hypostomus regani (N)	180–708	25	N	40	900	–	20	Mattias et al. (1998)
			H	30	1100	–	35	
Hypostomus regani (N)	300–600	20	N	25	400	110	–	Fernandes et al. (1999)
			H	15	600	130	–	
Hypostomus regani (N)	300–600	25	N	38	400	150	–	Fernandes et al. (1999)
			H	33	1500	190	–	
Hypostomus regani (N)	300–600	30	N	48	500	225	–	Fernandes et al. (1999)
			H	40	1500	250	–	
Hypostomus regani (N)	300–600	20	N	35	550	100	–	Nelson et al. (2007)
			H	14	600	100	–	
Hypostomus regani (N)	300–600	30	N	47	700	220	–	Nelson et al. (2007)
			H	40	1100	220	–	
Rhinelapis strigosa (N)	370–845	25	N	23	250	80	40	Takasusuki et al. (1997)
			H	21	1800	100	30	
Pterygoplichthys anisitsi (N)	88–600	25	N	52	734	117	20	Cruz et al. (2013)
			H	–	–	–	–	

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Temperature

The temperature influences O_2 consumption and respiratory responses of fish as they are ectothermic animals (Table 10.4, Fig. 10.16). Seasonal temperature changes occur gradually; however, fish may face water having different temperature and O_2 concentrations from the natural habitat when moving during the migration period for breeding or under flooding conditions. Temperature variation may result in an increase or decrease in the overall metabolic demand of the animal, as is evidenced by changes in VO_2 and ventilatory parameters. At high temperatures, *P. lineatus* (Barrionuevo and Fernandes, 1998; Fernandes et al., 1995a), *P. mesopotamicus* (Aguiar et al., 2002), and *O. niloticus* (Fernandes and Rantin, 1986b; Fernandes and Rantin, 1989; Maricondi-Massari et al., 1998) increase the aerobic metabolic demand (VO_2) as expected and exhibit V_G adjustments to maintain the O_2 extraction (Fernandes et al., 1995a; Rantin et al., 2007).

A sharp change to a new water temperature results in abrupt changes in VO_2, yet the acclimation occurs if the fish are maintained at the stable new temperature. Compensation for the acute effects on VO_2 is observed over hours and VO_2 reaches a stable level after a few days (Barrionuevo and Fernandes, 1998; Fernandes and Rantin, 1986b; Fernandes et al., 1995a; Rantin et al., 1985). Temperature changes concomitant with environmental hypoxia may alter the ability of a fish to regulate its O_2 uptake. In *P. lineatus*, for example, there is unchanged PcO_2 during gradual hypoxia after a 5°C change in water temperature; however, the exposure to temperatures of 10°C below or above that acclimation alters its capability to regulate O_2 uptake, changing the PcO_2 mainly when exposed to high temperatures (Table 10.4) (Fernandes et al., 1995a). A higher PcO_2 suggests a failure in the homeostatic mechanisms of the maintenance of the transport of O_2 from the water to the tissues. As the VO_2 changes, the V_G may show different control mechanisms that result in a greater increase in V_R than in f_R (Fernandes and Rantin, 1989). However, in *P. lineatus*, f_R increases more than V_R, implying a higher metabolic cost for respiration (Fernandes et al., 1995a).

In the lungfish *L. paradoxa*, the exposure at 15°C, 25°C, and 35°C showed a positive relationship between temperature, respiratory rate, and pulmonary ventilation (Amin-Naves et al., 2004, 2007). In loricariids, during progressive hypoxia without access to atmospheric air, the rapid transfer 5°C above or below the acclimation temperature changed the metabolic rate and capacity of gill ventilation, thus reducing the PcO_2 when compared to acclimated animals at each of those temperatures (Fernandes et al., 1999; Mattias et al., 1998; Nelson et al., 2007).

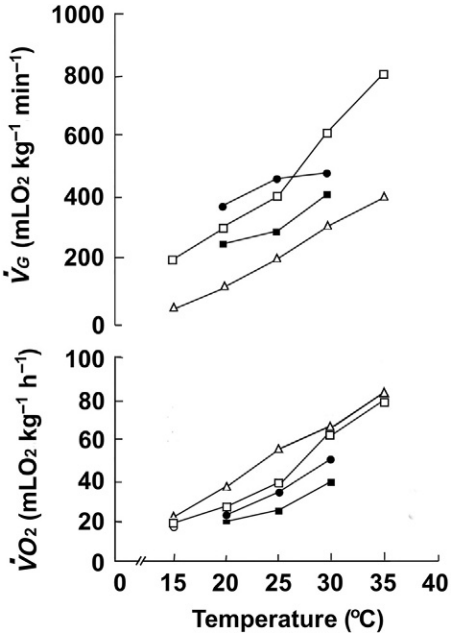

FIG. 10.16 Oxygen consumption (VO_2) and gill ventilation (VG) in normoxia in fish acclimated at different temperatures. *Oreochromis niloticus* (Δ), *Piaractus mesopotamicus* (□), *Hypostomus regani* (•), and *Rhinelepis strigosa* (▪). *(Data compilation from Aguiar, L.H., Kalinin, A.L., Rantin F.T., 2002. The effects of temperature on the oxygen uptake, gill ventilation and ECG waveforms in serrasalmid fish,* Piaractus mesopotamicus. *J. Therm. Biol. 27, 299–308; Fernandes, M.N., Rantin, F.T., 1989. Respiratory responses of* Oreochromis niloticus *(Pisces, Cichlidae) to environmental hypoxia, under different thermal conditions. J. Fish Biol. 35, 509–519; Fernandes, M.N., Sanches, J.R., Matsuzaki, M., Panepucci, L., Rantin, F.T., 1999. Aquatic respiration in facultative air-breathing fish: effects of temperature and hypoxia. In: Val, A.L., Almeida-Val, V.M.F. (Eds.), Biology of Tropical Fishes. INPA, Manaus, pp. 241–352; Maricondi-Massari, M., Kalinin, A.L., Glass, M.L., Rantin, F.T., 1998. The effects of temperature on oxygen uptake, gill ventilation and ECG waveforms in the Nile tilapia* Oreochromis niloticus. *J. Thermal Biol. 23, 283–290.)*

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Pollution

Water pollution has increased in recent years and the presence of xenobiotics in the aquatic environment causes considerable changes in water quality. Organic matter, metals, pesticides, hydrocarbons, and other substances accelerate the reduction of dissolved oxygen in the aquatic environment and/or induce biochemical, physiological, and/or morphological changes in the fish, resulting in changes in the physiology of the animal, including respiration.

Gills are the first organs to be affected by xenobiotics. Changes in gill tissue, such as defense responses and lesions caused by xenobiotic contact with the epithelium or uptake by the gills, alter their functions due to cellular and/or circulatory dysfunction (Mallat, 1985). Cellular hypertrophy, filament epithelium hyperplasia and lamellar epithelium, edema, and lamellar epithelial detachment are defense responses that increase the water-blood diffusion distance (Delunardo et al., 2013; Fernandes and Mazon, 2003; Mazon et al., 2002; Medeiros et al., 2017; Paulino et al., 2012, 2014). Proliferation of MRCs in the lamellar epithelium also increased the water-blood diffusion distance and may affect the gas exchange (Fernandes et al., 2013; Simonato et al., 2013). Mucus production reduces the rate of xenobiotic uptake but also reduces the diffusion of respiratory gases that can generate internal hypoxia (Ale et al., 2018). Necrosis reduces the gill surface irreversibly and causes blood congestion, aneurysms, lamellar epithelial rupture, and hemorrhages. These processes change the gill circulation that affects O_2 uptake from water and the ability to maintain the O_2 cascade when fish face environmental hypoxia (Fernandes and Mazon, 2003).

Exposure to the sublethal concentrations of trichlorphon for 96 h both decreased VO_2 and increased PcO_2 in the tropical fish *O. niloticus,* reducing its ability to survive under hypoxic conditions over a long time period (Thomaz et al., 2009). Exposure to mercury increased VO_2 and PcO_2 in *Brycon amazonicus,* and decreased VO_2 and increased PcO_2 in *H. malabaricus* (Table 10.4) (Monteiro et al., 2013). Hg exposures modulated the hypoxia-induced responses by affecting the ventilatory and heart adjustments. This resulted in impairment of the cardiorespiratory system of both species, which reduced survival under severe hypoxic conditions (Monteiro et al., 2013). Cyanobacteria toxin (100 μg MR-LR kg^{-1} body mass) induced changes in the ventilatory parameters by increasing V_G and V_R and reducing O_2 extraction in *H. malabaricus* under hypoxia, which may affect fish survival in such environmental conditions (Martins et al., 2019).

A reduction in f_R was observed in air-breathing fish when exposed to petroleum (Fernandes-Castilho et al., 2004; Val and Almeida-Val, 2004) while the exposure to the water-soluble fraction of petroleum containing poly-aromatic hydrocarbons increased the frequency of air breathers (Brauner et al., 1999).

Breath control

The unidirectional water flow on fish gills during respiration is achieved by a suction/force pump operated by the buccal and opercular ventilatory muscles, innervated by the cranial nerves. Under hypoxic conditions, fish may also use the hypobranchial pump for active jaw occlusion, operated by the hypobranchial muscles that are innervated by the hypobranchial nerve, which has branches of occipital nerves and anterior spinal nerves (Taylor et al., 2010). This same pump is used to ventilate the air-breather organs in the air breathing. (Taylor et al., 2010).

In the gills, the motor control of the branchial musculature and several sensory modalities (e.g., chemoreceptors, baroreceptors, nociceptors) was conducted by efferent (motor) or afferent (sensory) innervation. Gill innervation consists of branches of the glossopharyngeal and vague nerves, respectively the IX and X cranial nerves, which contain sensory and motor fibers (Morris and Nilsson, 1994; Nilsson, 1984). The posttrematic branch (ps) of the glossopharyngeal and the first pretrematic (pr) branch of the vagus enervate the first gill arch and are constituted by sensorial and motor fibers. The second, third, and fourth branches of the vagus also divide into pr and ps branches and enervate the other gill arches (Nilsson, 1984). These branches are constituted by sensorial and motor fibers, including fibers of the autonomic system of the sympathetic (sympathetic ganglion) and parasympathetic (cranial) chains. The sympathetic (CS) ganglia (IXS and $X_{1234}S$) are located near the outlet of the glossopharyngeal and the vagus in the wall of the buccal cavity. The sympathetic chain nerves are connected to the cranial nerves that form the sympathetic trunk of the vagus and enter the respective gill arches. The branchial nerve of the gill arch gives rise to a nerve bundle in the filament that continues distally and runs adjacent to the efferent filament artery, giving rise to a nerve plexus around this artery, innervating the superficial and interlamellar regions of the filament, and extending branches into the secondary lamellae (Jonz and Nurse, 2008). In the neotropical species *H. malabaricus* (traira) and *H. lacerdae* (trairão), there were no observed lamellar nerve fibers (Coolidge et al., 2008).

Many processes, such as the motor control of the branchial musculature and several sensory modalities (e.g., chemoreceptors, baroreceptors, nociceptors), are achieved by the efferent (motor) or afferent (sensory) innervation of the gills. The control of blood flow in the gills depends on the nervous and humoral/local system of the branchial vasculature as well

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as respiratory demand versus ion regulation, the known "osmorespiratory compromise." The distribution of blood flow in the gill vascular network is influenced by the catecholamines released by the sympathetic chain fibers. This action is mediated by β-adrenergic receptors in the arterioles that irrigate the lamellae and induce a vasoconstriction in the arterio-venous vascular system, favoring the arterial-arterial circulation. Studies carried out on three hypoxia-tolerant neotropical species (traira, jeju, and pacu) showed that humoral stress responses appeared in traira and jeju when the blood PO_2 was reduced to approximately 50%–60% of the normoxia values; these responses were not observed in *P. mesopotamicus* even in severe hypoxic conditions (Perry et al., 2004). Cholinergic control is exerted via vagal innervation and favors arterio-venous circulation.

Changes in ventilation and cardiac function occur concomitantly with vascular changes; both depend on the sensitivity of the chemoreceptors present in the gill system. The rapid detection of change in water and/or blood and cardiovascular and respiratory adjustments are vital for fish survival. These adjustments may occur due to the direct effect of O_2 availability on the different organs, but may also be induced by substances released by the endothelium (Burnstock, 1993a, b). Central and peripheral chemoreceptors (O_2 and CO_2/pH sensitive) evoke reflex responses in the cardiovascular and respiratory systems. Peripheral chemoreceptors were identified in fish gills and extrabranchial regions (Fritsche and Nilsson, 1989; Fritsche and Nilsson, 1993; Nilsson, 1984) and their distribution is not uniform among species. The presence of O_2-sensitive chemoreceptors was demonstrated in all gill arches of *Colossoma macropomum* (Sundin et al., 2000). Studies to determine the presence of receptors in gills and their distribution and action are done by bilateral or selective ablation of the gill branches of nerves IX and X, followed by alterations in PO_2 and PCO_2/pH in the external environment and blood. In the gills, two groups of receptors sensitive to changes in dissolved O_2 were identified: externally oriented receptors, sensitive to O_2 changes in the aquatic environment, and the internally oriented receptors, sensitive to O_2 dissolved in the blood (Burleson and Milson, 1993). Both types of chemoreceptors were responsible for the reflex responses that ensured efficient respiration by adjusting the ventilation/perfusion ratio on the respiratory surface. The O_2 receptors are responsible for triggering the ventilatory mechanisms by regulating the water flow over the gills and the reflex on heart rate, cardiac volume, and blood pressure, changing the blood flow through the lamellae; the internal receptors appear to be involved only in the ventilatory reflexes (Milsom, 1993).

In erythrinids, the distribution of chemoreceptors that initiate the ventilatory and cardiovascular responses varied between species. Bradycardic reflexes in response to environmental hypoxia depended on the stimulation of internal O_2 receptors in the first gill arch; increasing vascular resistance originates in gill and extrabranchial O_2 receptors. The increase in f_R originates in receptors that are exclusively located in the gills. However, increasing V_R involves extrabranchial O_2 receptors (Sundin et al., 1999). In trairão fish, external and internal O_2 chemoreceptors are distributed on the outer and inner surfaces of all gill arcs and initiate changes in the f_H value and ventilation amplitude while f_R is initiated by O_2 receptors on the surface of the first gill arch. The gradual exposure of trairão to hypoxia resulted in a 45% reduction in f_H and an increase of 50% in V_R and 32% in f_R (Micheli-Campbell et al., 2009). In the jeju fish, O_2 receptors involved in ventilatory and cardiovascular changes were present in all gill arches and the air breathing was initiated by a stimulation of receptors in the gill that responded to internal hypoxemia (Lopes et al., 2010).

In tambaqui, a species that uses ASR in environmental hypoxia (Val and Almeida-Val, 1995), the O_2 receptors that detect changes in blood and water, initiating bradycardia and increasing f_R, were found in all gill arches (Milsom et al., 2002; Sundin et al., 2000). In this species, extrabranchial O_2 receptors in the orobranchial cavity were oriented toward the alterations in the level of O_2 in the external environment and initiated the increase of vascular resistance, V_R, and the volume of the lower lip as a funnel for ASR (Florindo et al., 2006; Sundin et al., 2000). Gill receptors may also initiate such responses (Sundin et al., 2000). In pacu, the location and distribution of the extrabranchial chemoreceptors differed from those found in tambaqui, despite the similarity of the habitat and the close phylogenetic relation of these species. In pacu, the receptors are oriented to the external and internal medium, yet their location is still unknown (Leite et al., 2007, 2009). *C. mesopotamicus* has hidden pseudobranches; these structures can potentially be the sites of extra branchial receptors.

In traíra, the CO_2/pH receptors are exclusively in the gill arches and have a similar distribution of O_2 receptors. Traíra respond to hypercarbia, reducing their heart frequency and increasing their V_G value (Reid et al., 2000). Extrabranchial receptors may be involved in controlling the increase in V_R (Reid et al., 2000). In tambaqui, chemoreceptors are oriented to the external medium and are stimulated by PCO_2, initiating and increasing the cardiorespiratory responses to hypercarbia (Gilmour et al., 2005; Milsom et al., 2002). The breathing pattern (continuous, cycling, or episodic) appears to be determined by the activation of chemoreceptors (Reid et al., 2003).

In the Tetrapod, breath control is mainly related to an acid-base equilibrium and involves the central and peripheric chemoreceptors. In lungfish, which are considered a sister group of vertebrates and have functional lungs and reduced gills, there is evidence of the presence of central chemoreceptors (Sanchez et al., 2001) and cardiorespiratory interactions

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(Monteiro et al., 2018). The pulmonary ventilation and f_R in *L. paradoxa* increase with a reduction in the pH of the cerebrospinal fluid, suggesting that there is a possible central acid-base receptor like that of terrestrial vertebrates (Amin-Naves et al., 2007; Sanchez et al., 2001).

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Chapter 11

Nutrition and functional aspects of digestion in fish

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Introduction

Aspects of fish nutrition involving the physiological value of nutrients, the compartmentalization of digestive processes, and the functional aspects of digestion herein discussed focus on tropical freshwater species. Nutrition means studying all sets of actions ensuring the supply of nutrients required for the vital processes of an organism. These actions include feeding habits, food intake, digestion, absorption, nutrient metabolism, and release of wastes. Nutrition is also understood as an area of physiology intended to establish relationships between food intake and resulting growth, allowing a comparison of possible dietary ingredient use and metabolism, thus defining the nutritional requirements of an organism.

The interest in fish nutrition as a science is recent. Until the first half of the twentieth century, studies were mainly devoted to the anatomy of the digestive tract and to the physiology of the fish in nature. However, the development of fish farming brought about an urgent need to study the nutritional requirements of fish, given that diets can influence behavior, structural integrity, health, physiological functions, reproductive cycle, and growth (Guillaume and Choubert, 2001). Therefore, studying and defining qualitative and quantitative requirements of essential nutrients are fundamental

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for adequate formulation of fish feed. As a matter of fact, it has been long recommended that nutritional studies on the growth of fish should consider the presence of energy sources, essential amino acids, vitamins, and minerals in the diets (Weatherley and Gill, 1987).

The nutritional value of a given diet is assessed first by the presence of required and catalytic elements (minerals and vitamins), an abundant supply of auxiliary food (water), and an adequate balance between energy and plastic nutrients (carbohydrates, lipids, and proteins). In addition, the nutritional value of a diet is also given by the digestibility of ingredients and the absorption of the nutrients by animals because diets are not considered actually utilized by animals until absorbed and made available to the metabolism. Finally, the nutritional composition of a diet can affect the production of digestive enzymes and the nutrient transporting density in cell membranes, thus affecting the digestive and absorptive abilities of animals.

Metabolic oxidation of carbohydrates, lipids, and proteins releases from chemical bonds the energy that is primarily used for maintenance of the basal metabolism, and later becomes available for growth. The nutritional balance of a diet is thus essential because an energy deficiency can lead to the use of dietary protein as an energy source instead of for growth, and excess dietary energy can reduce feed consumption, which hampers the intake of the required minimum amounts of proteins and other nutrients. Exceedingly high levels of dietary energy can also lead to the deposition of large amounts of body fat by fish, harming flesh quality. In addition, portioning dietary energy for the basal metabolism and growth is affected by factors such as temperature, oxygen availability, feed frequency, voluntary or involuntary locomotion, basal metabolic rate, specific dynamic action, and hormone levels (Kapoor et al., 1975).

Nutrients

Food is the natural source of nutrients for the sustainability of the structures and functions of living organisms. Dietary nutrients can be classified into macro- and micronutrients, according to their quantitative proportion in diets. Three major macronutrients are considered in the composition of diets: proteins, carbohydrates, and lipids. The micronutrients considered include vitamins and minerals.

Macronutrients

Proteins

Proteins are the building blocks of muscle cells and organs. They occur in a great array of forms and their nutritional value depends on their amino acid composition (proportion). Proteins of higher nutritional value have a higher content of essential amino acids, that is, amino acids that are not synthesized by animals—arginine, histidine, leucine, isoleucine, methionine, phenylalanine, threonine, tryptophan, and valine. Based on their nutritional value, protein sources for fish feeds are classified as either first- or second-class proteins (Weatherley and Gill, 1987).

Animal proteins, particularly fishmeal, have higher nutritional values than plant proteins because they match amino acid requirements and the muscle protein profile (ideal protein) of most animals, particularly fish. Studies have been carried out toward the replacement of fishmeal by plant protein sources, such as soybean meal, that present high nutritional value, reasonable cost, and abundant supply. However, replacing dietary animal protein sources by plant protein is not recommended for some fish because it may result in reduced feed conversion and growth as a consequence of deficiency in some amino acids.

The use of a given dietary protein depends directly on the functional, biochemical, and genetic characteristics of a given fish species; the age of organisms; the range of environmental factors (pH, dissolved oxygen, and ammonia levels); the amino acid contents of dietary protein (food and feeds); the inclusion levels of dietary protein; and the digestible usable dietary energy levels. Dietary protein requirements are also affected by the presence of other essential nutrients such as vitamins and minerals. In addition, the amino acid contents (proportions), the structural configuration, the digestibility (including digestibility of other dietary ingredients), and the possible presence of growth inhibitors or antinutritional factors in the protein source will determine the use efficiency and the relative value of a dietary protein.

Dietary protein requirements of fish decrease with age, i.e., arrival at sexual maturity, as registered for example for the channel catfish, *Ictalurus punctatus*, which requires 35% and 25% dietary protein in the juvenile and adult phases, respectively; similar quantities are required by salmonids and carp, *Cyprinus carpio*, and by tilapia, *Oreochromis niloticus* (NRC, 2011). Fingerlings have higher dietary protein requirements than juveniles, and these decrease with growth (age). The dietary protein requirements in the early growth stages are related to the relatively rapid increase of most tissues as they

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grow with positive allometry, in addition to their relatively higher metabolic conversion (Weatherley and Gill, 1987). The dietary protein requirements for maximum growth have been estimated in several species throughout the juvenile phase. However, such requirements are often overestimated (Wilson, 2002), and because the excess amino acids cannot be stored, they are used as alternates to carbohydrates and lipids as an energy source.

Amino acids are protein building blocks, and there are some amino acids that fish are unable to synthesize, the so-called essential amino acids. Deficiencies of essential amino acids in the diets hamper growth rates and give rise to deficiency syndromes, such as cataracts and anatomical deformities. Therefore, such amino acids should be included in the diet of fish (Weatherley and Gill, 1987). Intaken proteins are "processed" via two metabolic routes: (i) catabolism, producing energy for maintenance and (ii) anabolism, engaged mainly in the protein synthesis de novo. For these purposes, the amino acid relative contents (proportions) of a diet are crucial (Hepher, 1989). Growth is related to food intake and, when it is understood as protein deposition (tissue accretion), it will be determined by the relationship between the rates of synthesis and the degradation of proteins (Millward, 1989). This relationship may be linked to the nitrogen balance, which may be positive (i.e., intake—nitrogen excretion >0) or negative (Carter and Houlihan, 2001). Because of its high energy cost, protein synthesis has a major metabolic impact. During the growth phase, protein synthesis may be responsible for the use of 42% of the total dietary energy intake, becoming the physiological process of highest energy demand in juvenile fish. However, only a relatively small portion of the protein ingested in a meal is retained as a body component, contributing to animal growth (Carter and Houlihan, 2001).

Another aspect to consider regarding proteins as macronutrients in fish diets is the limit to which the species is able to use them for growth. After deamination of its amino component, excess amino acids are stored as lipids, i.e., an energy source (Hepher, 1989). Ammonia, known to be harmful to fish health at high environmental levels, is produced in response to the use of proteins as an energy source (McGoogan and Gatlin, 2000). The excretion of ammonia by fish promotes eutrophication of the water when the medium is imbalanced. Therefore, excess dietary protein results in poor growth performance, deterioration of the water quality, and increased production costs. However, ingestion of proteins to meet energy requirements is a common strategy among fish, and that may be lessened inasmuch as the protein fraction is replaced by nonprotein energy sources (Weatherley and Gill, 1987). Moreover, the imbalance between dietary energy sources and dietary protein contents may hamper the intake and use of dietary protein for growth (Ellis and Reigh, 1991). Therefore, it is vital to the development and growth of fish the balance between dietary protein fraction and energy content, also provided by lipids and carbohydrates (Carter and Houlihan, 2001).

Carbohydrates

Carbohydrates play important metabolic roles, such as providing glucose as an energy source to the tissues, precursors of nucleic acids, and mucopolysaccharides. Some carbohydrates have agglutinating properties, relevant in the production of diets. However, the use of carbohydrates as a partial surrogate of proteins as an energy source is still controversial in fish nutrition (Lundstedt et al., 2004). The use of carbohydrates varies to a great extent among fish, and many questions remain to be answered and understood. In several fish, carbohydrates do not seem to have the same relevance they have in the metabolism of mammals, being practically neglected and bypassed as a dietary energy source. However, most enzymes involved in the carbohydrate metabolism in mammals are also reported for fish.

The use of dietary carbohydrates vary according to fish food habits, environmental conditions, and the type of carbohydrate, among other factors (Honorato et al., 2010; Moro et al., 2016). Such variability also seems to be associated with the complexity of the molecules. The ability to digest carbohydrates varies to a great extent among species and reflects the anatomical and functional differences of the gastrointestinal tract and associated organs as well as the environmental temperature (Krogdahl et al., 2005). The capacity of using carbohydrates by neotropical fish tends to be much greater than that of marine and cold water fish. Neotropical fish can utilize complex carbohydrates, such as starch and dextrin, more efficiently whereas temperate and marine fish are more suited to use mono and disaccharides. Because of the scarcity of carbohydrates in the aquatic environment, the metabolism and metabolic systems of fish seem to be better adapted to the use of proteins and lipids as energy resources. However, herbivorous and omnivorous fish can digest and metabolize carbohydrates relatively well when compared with carnivorous species such as salmonids (Weatherley and Gill, 1987; NRC, 2011; Wilson, 2002). Although there is a scarcity of studies on the specific requirements, both quantitative and qualitative, of carbohydrates for the many farmed fish species, the inclusion of this macronutrient in their diets is done because of the important role it plays as a lower-cost energy source in relation to dietary protein (McGoogan and Gatlin, 2000), and also because dietary carbohydrates bring smaller environmental problems.

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Lipids

Lipids are the main energy source for animals (9.4 kcal g^{-1} of crude energy) and their main role is to generate metabolic energy as ATP via β-oxidation. Lipids comprise chiefly triacylglycerols, fatty acid esters, and glycerol; other lipid compounds, chemically distinct from triacylglycerols, are steroids and waxes. Lipids provide greater palatability to food, serve as a vehicle for absorption of lipid-soluble vitamins and steroids, and play an important role in the structure of biological membranes in the form of phospholipids and steroid esters (Hepher, 1989). Lipids are also the major source of metabolic energy for reproduction in fish (Halver and Hardy, 2002). In addition, lipids provide the organism with essential fatty acid, a key nutrient for good overall development (Chou et al., 2001).

In nature, lipids are present in both animals and plants and are stored primarily as triacylglycerols and waxes. However, in freshwater organisms, waxes are not found in large amounts, whereas triacylglycerols are the main component of lipids, both ingested and stored. The natural diets of many fish contain large amounts of long-chain polyunsaturated fatty acids (PUFAs) and, in contrast with terrestrial animals, their lipid composition also contains high levels of PUFAs, predominantly of the ω-3 series (linolenic acid) (Weatherley and Gill, 1987). Triacylglycerols can be stored in various organs, mainly viscera, liver, and muscles, corresponding to 15%–45% of the visceral weight. High concentrations of lipids contribute approximately 10% of all body weight in fish, but there is little free fatty acid in their tissues (Weatherley and Gill, 1987).

Dietary lipid absorption in fish is a process similar to that of mammals. After intraluminal hydrolysis, dietary lipids are captured by intestinal epithelial cells after diffusion of the micellar form of monoglycerides and fatty acids. Fatty acids are also found as phospholipid and steroid constituents in the cells (Weatherley and Gill, 1987).

The current trend in fish nutrition is to increase dietary lipid content to spare protein, improving feed conversion and lessening nitrogen excretion. Lipids are highly energetic and readily metabolized by fish, especially carnivores (NRC, 2011). They serve as important sources of energy, especially for marine species, which usually have a limited ability to use carbohydrates as an energy source (Chou et al., 2001). Lipid content between 10% and 20% of the diets (dry weight) is sufficient to drive the dietary protein fraction through growth without excessive fat deposition in the carcass (Cowey and Sargent, 1979). In addition, the precise dietary requirement on lipids depends on the level of dietary protein and, in some cases, on the carbohydrate level (Watanabe, 1982).

Micronutrients

Vitamins

Vitamins are organic compounds considered essential to bodily functions, as they play important roles in the cell biocatalytic processes. Several researchers characterize vitamins as essential given the inability of organisms to synthesize them. However, niacin, for example, can be synthesized from tryptophan, cholecalciferol from cholesterol under sufficient ultraviolet radiation, and retinol from certain precursors (mainly β-carotene) supplied by the diet. The concept of the inability of synthesis is thus inapplicable to all vitamins. In contrast, there are compounds, such as carnitine, that have not been included in the list of vitamins because they can possibly be synthesized, even though the synthesis rate is insufficient to supply dietary requirements (Guillaume and Choubert, 2001). The synthesis of vitamins may be distinct among species; for example, many vertebrates can synthesize ascorbic acid from glucose but the primates and man do not have this ability. In addition, the active intestinal biota of animals may have some bacteria capable of synthesizing the vitamins of groups B and K, dispensing with the use of supplements. However, this fact is not reported in fish, which have limited activity of intestinal biota and need to take required vitamins from food. There are also substances (natural or synthetic) called antivitamins that can prevent the intestinal absorption of vitamins, acting as antagonists or hydrolyzing the micronutrient (Guillaume and Choubert, 2001).

Vitamins are classified according to their solubility into two main groups: group B (water soluble), which numbers 11 (Table 11.1), and group A (fat soluble), which numbers 4 (Table 11.2). It is very difficult to specify a particular function for each group. Group B encompasses molecules that are fundamental to the intermediate metabolism of plants and animals (usually coenzymes) while vitamins of group A play many physiological roles and sometimes are not detected or present in plants. In regard to the nutritional aspects of vitamins, their biological role, and the required quantities, there are small differences between superior vertebrates and fish. Two vitamins, calciferol and vitamin K, seemingly play a minor nutritional role in fish, whereas vitamins E and C are more relevant to fish than to higher vertebrates (Guillaume and Choubert, 2001). An abridged overview of vitamins and their major physiological roles is presented in Tables 11.1 and 11.2.

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TABLE 11.1 Water-soluble vitamins and their functions

Vitamin	Active form	Role
Thiamine (B_1)	Thiamine pyrophosphate (TPP); Thiamine triphosphate (TTP)	Carbohydrate metabolism, C1 carrier group (aldehyde), glyceraldehyde groups, neurotransmission
B_2 (riboflavin)	Riboflavin phosphate or flavin mononucleotide (FMN) or FAD	Key role in oxidation-reduction reactions, catabolism of fats, amino acids, and purines, reduction of glutathione, maintenance of erythrocyte integrity, activation of pyridoxamine, oxidative processes
B_6 (pyridoxine, pyridoxal, pyridoxamine)	Pyridoxal phosphate	Metabolism of amino acids, formation of biogenic amines. Synthesis of pancreatic enzymes and glycogen metabolism
B_{12} (cyanocobalamin)	Cyanocobalamin, hydroxocobalamin, methylcobalamin	Isomerization reactions, transmethylation, folate metabolism, antianemia, cholesterol metabolism, purine and pyrimidine synthesis
Biotin (vitamin H)	Biotin	Carboxylation and decarboxylation reactions, carbohydrate metabolism, lipogenesis, *n*-odd fatty acid catabolism, leucine catabolism, citrulline, purine and pyrimidine synthesis
PP (niacin)	Nicotinic acid, nicotinamide, NAD e NADP	Oxygen reductive processes, carbohydrate, lipid and amino acid metabolism, lipid biosynthesis, steroid and phenylalanine metabolism
Pantothenic acid		Lipid metabolism, C_2 unit transporter, decarboxylation processes, cholesterol biosynthesis, and mucopolysaccharides
Folic acid	Tetrahydrofolate	Transfer of C_1 units, amino acid interconversion, protein synthesis, purine and pyrimidine synthesis, red cells (antianemic), blood glucose regulation, egg hatching, and immune system
Ascorbic acid		Redox processes, H_2 donor in hydroxylation reactions, collagen and red cell maturation, synthesis of catecholamines and carnitine, catabolism of phenylalanine and tyrosine, neutralization of free radicals, absorption of iron and other bi- and trivalent ions, vitellogenesis, degradation of xenobiotics

Compiled from: Guillaume, J., Choubert, G., 2001. Digestive physiology and nutrient digestibility in fishes. In: Guillaume, J., Kaushik, S., Bergot, P., Métailler, R. (Eds.), Nutrition and Feeding of Fish and Crustaceans, UK, pp. 27–58; Hepher, B., 1988. Nutrition of Pond Fishes. Cambridge University Press, Cambridge, England; Steffens, W., 1989. Principles of Fish Nutrition. Ellis Harwood Ltd., New York, NY.

TABLE 11.2 Fat-soluble vitamins and their functions

Vitamin/ requirements	Active forms	Role
A	Retinoic acid, retinol, retinal	Coenzyme in the processes of vision (rhodopsin and iodopsin), hormonal mechanisms (growth vitamin), mRNA induction, specific protein synthesis, cell differentiation, mucus secretion, immune response, reproductive functions, protection against tumors
D (calciferol)		Regulation of phosphorus-calcium metabolism, mobilization of Ca^{2+} bone, intestinal and renal absorption of Ca^{2+}, growth, cell differentiation, and immune system
E (tocopherol)	α-Tocopherol	Antioxidant function, protection of vitamin A, HUFAs, carotenoids and coenzyme Q against oxidations, immune processes, prostaglandin synthesis, reproductive functions
K (naphthoquinone)		Blood coagulation, prosthetic group of a carboxylase of glutamic acid, transport of calcium, electron-charger

Compiled from: Guillaume, J., Choubert, G., 2001. Digestive physiology and nutrient digestibility in fishes. In: Guillaume, J., Kaushik, S., Bergot, P., Métailler, R. (Eds.), Nutrition and Feeding of Fish and Crustaceans, UK, pp. 27–58; Hepher, B., 1988. Nutrition of Pond Fishes. Cambridge University Press, Cambridge, England; Steffens, W., 1989. Principles of Fish Nutrition. Ellis Harwood Ltd., New York, NY.

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Nutrition Supplements

Also known as dietetic or dietary supplements, nutritional supplements are compounds that animals can synthesize at a very slow pace under certain conditions. Among them are the orotic acid (vitamin B_{13}), ubiquinone, pyrroloquinoline quinone, lipoic acid, and carnitine (Guillaume and Choubert, 2001) (Table 11.3).

Minerals

Minerals are micronutrients playing plastic and regulatory functions in organisms, such as ionic and structural equilibrium functions; they also act as metabolic cofactors. Specific concentrations and functional forms of minerals need to be kept within narrow variations for the proper functioning of the body's metabolic activities, and this is done through homeostatic mechanisms. Minerals are responsible for several functions, such as skeletal formation, maintenance of colloidal systems, and regulation of the acid-base balance and biologically important compounds such as hormones and enzymes.

Mineral deficiencies can cause biochemical, structural, and functional pathologies that depend on factors such as the extent and degree of deprivation. The physiological importance of minerals is well documented in humans and some other mammals and poultry. However, many aspects of trace mineralization, function, and bioavailability remain unclear. The requirements on all trace elements have not yet been described in fish. In addition, information on fish mineral requirements is fragmented, possibly because these micronutrients are required in minute amounts ($\sim$100 mg kg^{-1} of dry diet). Nevertheless, the ingestion and assimilation of minerals are obligatory to normal growth, just as their excess in the diet can cause toxicity to the fish. Thus, maintaining a precise body balance of trace element levels depends on the integration of the uptake, storage and excretion mechanisms (Watanabe et al., 1997).

The use of dietary minerals depends on their bioavailability, and the efficiency with which the animals use them can be influenced by factors such as: the amount available, the size and shape of the particles, the digestibility, the synergistic or antagonistic interactions between the nutrients of the diet, the pathophysiological conditions of the animal, the concentration in the water and the species under consideration. Among these factors, those related to chemical status stand out because the element may adopt different molecular forms, valence states, and ligands when ingested from different diets. In addition to all diet-related mechanisms, several environmental aspects influence the bioavailability of the mineral (Watanabe et al., 1997; Lal, 2002).

Dietary mineral supplementation for either freshwater fish or salt water fish is very important. The external freshwater environment is very hypotonic in relation to the internal *milieu*. The difference in osmotic pressure leads to a passive net loss of salts and water absorption through the skin, with renal excretion characterized by large volumes of much diluted urine (around 100 mL kg^{-1} day^{-1}). At the same time, the loss of sodium is compensated by an increase in the efficiency of the sodium pump in the gills. The salt water external environment is hypertonic in relation to the internal medium of fish. The osmotic pressure leads to dehydration and there is a significant absorption of salts via the gills. Saltwater fish actually drink large amounts of water only to maintain the osmotic balance, which is rich in salts and ions. Excess ions are eliminated by the kidneys (divalent ions), gills (mainly sodium and chloride), and intestine (divalent ions). Thus, the chance of mineral intake by saltwater fish is much higher than that by freshwater fish because the minerals dissolved are much more concentrated and almost constant, and fish are bound to drink water continuously to maintain the osmotic balance (Guillaume and Choubert, 2001).

Seven macronutrients (Table 11.4) and 15 trace elements are known to play a defined physiological role in many animals. However, their dietary requirement in fish is known only to 11 of them (Table 11.5). For fish, little is known about

TABLE 11.3 The quasivitamins and their functions

Vitamin	Active Form	Role
Inositol	Phosphatidylinositol	Structural role, lipotrophic factor, involved in the translation of signals from certain metabolic processes, by the generation of secondary messengers (IP_3), the only "vitamin" with energy value being degraded as a carbohydrate, component of phospholipids and lipid metabolism
Choline		Nerve transfer by cholinergic fibers (acetylcholine), phosphatidylcholine composition, lipotrophic, antihemorrhagic, lipid transport, methionine-sparing because it is betaine precursor (methyl group donor)

Compiled from: Guillaume, J., Choubert, G., 2001. Digestive physiology and nutrient digestibility in fishes. In: Guillaume, J., Kaushik, S., Bergot, P., Métailler, R. (Eds.), Nutrition and Feeding of Fish and Crustaceans, UK, pp. 27–58; Hepher, B., 1988. Nutrition of Pond Fishes. Cambridge University Press, Cambridge, England; Steffens, W., 1989. Principles of Fish Nutrition. Ellis Harwood Ltd., New York, NY.

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TABLE 11.4 Macrominerals: sources and role

Mineral	Sources	Role
Calcium (Ca^{2+})	Animal flours, 50%; from the water	Development and growth of skeletal structures, maintenance of osmotic balance, muscular activity and functioning of the nervous system, blood coagulation, regulation of hormonal secretions
Phosphorus (P)	Low environmental absorption	Formation of hard tissues (bones and teeth), interrelation with Ca^{2+}, metabolic processes, connective tissue, phospholipids, nucleic acids, energy-rich compounds, etc.
Magnesium (Mg^{2+})	Partially found in water; correlation with dietary protein	Fundamental constituent of bone tissues, cofactor of enzymatic reactions related to phosphorus (pyruvate kinase, phosphokinase, phosphatase, ATPase) involved in osmoregulation, protein synthesis, and growth
Sodium (Na^{+}), Chloride (Cl^{-}), Potassium (K^{+})	Ambient water	Responsible for extracellular (Na^{+} and Cl^{-}) or intracellular (K^{+}) equilibrium; in osmoregulation by ATPase Na^{+}-K^{+} and chloride cells vital importance in gastric (Cl^{-}) digestion
Iron (Fe^{2+}/Fe^{3+})	Animal foods; vitamin C affects availability	Oxidation-reduction reactions, cellular respiration, hemeproteins, ferroprotein, is one of the primary metals involved in lipid oxidation; Fe^{2+} catalyzes the formation of hydroxyl peroxides and peroxides

Compiled from: Guillaume, J., Choubert, G., 2001. Digestive physiology and nutrient digestibility in fishes. In: Guillaume, J., Kaushik, S., Bergot, P., Métailler, R. (Eds.), Nutrition and Feeding of Fish and Crustaceans, UK, pp. 27–58; Hepher, B., 1988. Nutrition of Pond Fishes. Cambridge University Press, Cambridge, England; Steffens, W., 1989. Principles of Fish Nutrition. Ellis Harwood Ltd., New York, NY.

TABLE 11.5 Microminerals: sources and role

Mineral	Sources	Role
Manganese (Mn^{2+})	Fish meal	Metabolism of lipids, proteins, and carbohydrates as an enzymatic cofactor and as an integral part of metalloenzymes (decarboxylases)
Iodine (I_2)	Water environment, plants/ marine animals, diet	Present in thyroid hormones (T_3 and T_4), cellular oxidation, neuromuscular control, circulatory dynamics, nutrient metabolism, and growth
Chromo (Cr^{3+})	Animal protein, oil seeds, and vegetables	Metabolism of carbohydrates and lipids, cofactor in the insulin activity
Sulfur (S)	Animal protein	Integral part of collagen, involved in detoxification of aromatic compounds
Cobalt	Animal protein	Component B_{12} (cyanocobalamin) prevents anemia and is involved in the metabolism of C_1 and C_3
Zinc (Zn^{2+})$^{(A)}$	Animal protein, cereals, and wheat	Essential to the structure and function of insulin, cofactor of carbonic anhydrase
Copper (Cu^{2+})$^{(B)}$	Plant and animal proteins	Activity of enzymes (cytochrome oxidase, superoxide dismutase, lysyloxidase, dopamine hydrolase and tyrosinase, ceruloplasmin) facilitates the absorption of other trace elements Fe^{2+} or Zn^{2+}
Molybdenum (Mo)	Vegetables, cereals, and viscera	Cofactor of xanthine oxidase, hydrogenase, and reductase
Fluorine (F^{-1})	Animal protein and water environment	Component of the bone apatite fraction

Compiled from: Guillaume, J., Choubert, G., 2001. Digestive physiology and nutrient digestibility in fishes. In: Guillaume, J., Kaushik, S., Bergot, P., Métailler, R. (Eds.), Nutrition and Feeding of Fish and Crustaceans, UK, pp. 27–58; Hepher, B., 1988. Nutrition of Pond Fishes. Cambridge University Press, Cambridge, England; Steffens, W., 1989. Principles of Fish Nutrition. Ellis Harwood Ltd., New York, NY.

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the importance of other trace elements, such as molybdenum, fluorine, vanadium, etc. Both the signs of deficiencies and the nutritional requirements of these elements are not yet well established (see NRC, 2011).

Digestion in fish: general features

The ingested nutrients undergo digestion and absorption before being transformed into the metabolic routes. Digestion is a coordinated combination of physical, chemical, and enzymatic processes that begins when food is ingested and ends with dejection. Physical processes include (as a rule) chewing, crushing, pulsating, and chyme churning. Chemical processes begin with the secretion of enzymes in the mouth and substances such as hydrochloric acid in the stomach or bicarbonate in the intestine. These secretions have an active participation in the hydrolysis process of the diet components. The enzymatic processes are more specific because they are aimed at protein hydrolysis of carbohydrates, lipids, and nucleic acids, involving a large number of enzymes (Halver and Hardy, 2002). As a rule, proteins are hydrolyzed to free amino acids or short peptide chains, carbohydrates are hydrolyzed to simple sugars, and fats into fatty acids and glycerol.

The main digestive tract sections in fish are the mouth, oral cavity, esophagus, stomach, intestine with pyloric cecum and related organs (liver, gallbladder, and pancreas), rectum, and anus (Corrêa, 2002). This division presents many adaptative modifications of structure and function, both related to the evolutionary position of the species and to the feeding habits. The stomach, intestine, pancreas, and liver produce digestive enzymes, bile (bile salts), and hormones that control the digestive biochemical process. Accessory structures such as the pyloric cecum, the blind diverticula annex to the proximal intestine, can be noticed in several species and have a secretory and absorption function (Buddington et al., 1997). The morphology of the digestive tract of teleosteans has been extensively studied and described in detail regarding the function of many anatomically specialized structures in relation to different adaptations of the species' feeding habits.

Morphological features of the digestive tract

The wall of a fish's digestive tract is histologically similar to that of higher vertebrates, comprising four basic layers: (i) mucosa built up of columnar epithelial cells, supported by loose connective tissue, and forming large undulated folds; (ii) submucosa, which is a layer of connective tissue that gives support to the mucosa; (iii) muscular tunica, comprising two muscle layers—longitudinal or external and transverse or internal circular—that drive the peristaltic movement; and (iv) serous, formed internally by loose connective tissue and externally by a layer of pavement epithelial cells (mesothelium), ultimately enveloping the gastrointestinal tract. These layers may vary considerably in the different sections and are not necessarily present in all organs of the digestive tract; they may also have additional structures or subdivisions.

Cells of the mucous epithelium are differentiated along the digestive tract according to the implicit function: absorption cells (intestine), secretory (stomach and intestine), or mucous membranes (along with the entire digestive tract displaying protective function) (Takashima and Hibiya, 1995). Numerous enzymes related to the digestive and absorption processes are located in the columnar epithelial cells along the striated border, providing them with the function of absorbing food particles and molecules as well as the digestive function.

The columnar cells of the mucosal epithelium have very developed Golgi apparatus with a polysaccharide synthesis function and a granular endoplasmic reticulum that produces the proteins that make up the secretion. Mucus has the protective function because it reduces the ability of bacteria to enter the enterocytes, also protecting them against possible physical and chemical damage (Buddington et al., 1997). It also helps in lubrication during the passage of food, provides enzymatic cofactors, or may contain digestive enzymes per se (Kapoor et al., 1975).

Functional features of the digestive tract

Fish have the simplest or less differentiated digestive system among vertebrates, actually in conformity with their position on the evolutionary scale (De Silva and Anderson, 1995). On the other hand, fish present a quite variable morphology of the digestive tube, which illustrates the diversity of diets and feeding behaviors covering the various trophic niches. Fish, on account of presenting an external anatomy of the digestive tract usually differentiated, are known for two main characteristics: morpho functional evolution and phenotypic adaptation to diet (Guillaume and Choubert, 2001).

The anatomy of the digestive tract in the most primitive fish families is homogeneous but varies in the most recent groups. Elasmobranchs present a particular type of digestive tract in which the stomach is well differentiated, presenting a short intestine with a unique structure, the spiral valve, which has a high capacity for absorption. The most recent families present high phenotypic plasticity as related to the feeding habit, such as the presence or absence of the stomach, different

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extents of the esophagus and intestine, intestines with different fold shapes, and a variable number of pyloric ceca (ranging from 9 to 1000). Despite these differences, some characteristics are conserved among groups: (i) the esophagus always presents striated musculature, allowing animals the possibility of regurgitation; (ii) the intestine resembles that of birds or the small intestine of mammals, with some differentiation similar to the duodenum, jejunum, and ileum, although no true colon can be defined; (iii) the intestinal wall has only three layers: the mucosa, muscular, and serous; (iv) the intestine villi are not well differentiated and look more like pseudovilli or folds; (v) enterocytes of some fish preserve endocytosis throughout life (similarly to newborn mammals); and (vi) the profile of digestive enzymes is relatively similar to that of the higher vertebrates (Guillaume and Choubert, 2001).

The digestive structures of the fish digestive tract reflect the variation of the nutrient sources ingested as food, ranging from short and simple to complex, closely resembling that of ruminants. Considering the long evolutionary history of fish as a group and the highly diverse ecological niches the group dwells in, their gut diversity seems to exceed that of other vertebrates (Krogdahl et al., 2005). The anatomical characteristics of the digestive tract are closely dependent on the food source and quality, the habitat characteristics, the nutritional status, and the individual development. These characteristics are observed especially in the gastrointestinal tract through adaptations and modifications (Seixas Filho et al., 2001).

The characteristics of the fish digestive tract determine the potential of assimilation of dietary nutrients. Differences in productive performance (growth) between species more likely result from differences in the characteristics of their digestive tracts and the efficiency with which the ingested food is digested and assimilated (Weatherley and Gill, 1987). The ability of fish to process the food also depends on specific characteristics such as the enzymatic profile of the alimentary canal and the adaptive characteristics of these enzymes. The efficiency of food absorption and feed conversion may also be dependent, besides the availability of digestive enzymes, on the transepithelial transport capacity in the digestive tract (Jobling, 1995). Thus, clear information on diet, feeding habits, and ecological niche is necessary to understand the digestion strategy of any given species.

Sections of the digestive tract

Not all fish have a fully differentiated digestive tract, either morphologically or functionally. However, it is possible to separate differentiated structures in most species.

Mouth and pharynx

The digestive process begins in the mouth and pharynx. The initial digestion is mechanical and, so far, no reports on the digestive activities in the fish mouth could be located. Because of the noncompressive nature of water and the presence of gills, the mouth has a complex function. It is used by the animal for the ingestion of water and food and therefore requires perfect coordination between the jaws, the tongue, the roof of the mouth, the gill arches, and the operculum (Guillaume and Choubert, 2001).

Certain fish have either teeth in the oral cavity to crush or lacerate food, and/or pharyngeal plaques to grind and crush (De Silva and Anderson, 1995). In addition, the teeth can be used for capture and seizure of the prey or for filtration. Mucous cells or mucous glands may still be present in the mouth and pharynx, but the most abundant secretion of mucus occurs in the esophagus. Mucus lubricates large food particles to aid in swallowing (Weatherley and Gill, 1987).

There are no salivary glands in the fish mouth. However, there are other organs outside the mouth formed by chemoreceptor cells. These cells are postulated to be olfactory, although the distinction between the olfactory and gustatory senses is overshadowed in the aquatic environment (Guillaume and Choubert, 2001).

Esophagus

The esophagi of fish are usually small and broad and present many mucous cells compared to the stomach and intestine. They also differ from other structures by the presence of striated musculature able to voluntary contract, which allows fish, when needed, to regurgitate ingested food of unsuitable size and/or texture or that is unpalatable. Some freshwater species present a cardiac sphincter at the esophagus-stomach junction likely to minimize water ingress (Guillaume and Choubert, 2001). The esophagus opens into a true stomach in most carnivorous fish, which differs from the esophagus regarding the composition of the walls and glands in the mucosa (Weatherley and Gill, 1987). A study on the activity of carbohydrates and proteases in the esophagus of the milkfish, *Chanos chanos*, for instance, reported lipolytic activity in the esophagus of the species, an indication that this structure plays a more active role in the digestion process of milkfish (Borlongan, 1990).

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Stomach

The fish stomach has a very distinctive morphology, going from a single, undifferentiated tube to an elongated, blind-end bag (when full) to a well-differentiated pouch. The stomach of fish can also be arched in a Y- or J-shaped form. In any case, longitudinal muscle fibers predominate in the stomach of fish. In carnivorous fish that engulf their whole prey, the stomach is capable of great distension. Despite such anatomical differences, the histological structure of the fish stomach is very conserved among species, and differs very little from the superior vertebrates regarding functionality. The stomach of fish can be divided into a proximal and a distal region, each one presenting specific features regarding the number of villi and cellular covering as well as the secretion patterns of zymogens, hydrochloric acid, hormones, or mucus in the endodermal epithelium. Mucus-secreting cells are found only in the distal part. Usually, the stomach is separated from the intestine by a pyloric sphincter or valve (Guillaume and Choubert, 2001).

The stomach of some herbivore fish has no secretory function. The pyloric region is morphologically (hardened walls) and functionally (crushing function) similar to the gizzard of birds. The fish stomach has a pH between 2.0 and 3.0, thus allowing the denaturation of proteins and the beginning of hydrolysis. This also elicits an antibacterial environment. However, the pH may reach 5.0, especially in marine fish that continuously ingest water with pH 8.0 (Guillaume and Choubert, 2001). The increase in the production and secretion of HCl may occur either by stimulating mechanoreceptors (gastric distension) or chemoreceptors (peptides, amino acids) in the stomach wall (Jobling, 1995; Lovell, 1988). In addition to mucosal cells, gastric glands with fundic cells secrete HCl and pepsinogen, particularly under control of the vagus nerve (Weatherley and Gill, 1987). There appears to be a direct relationship between gastric secretion and temperature. When the temperature increases by 10°C, a three- to fourfold increase in the digestion rate may be registered until the optimal temperature is reached (Kapoor et al., 1975). Digestion rates also vary interspecifically and according to the amount and type of food ingested (Weatherley and Gill, 1987). Regarding the mechanism of gastric acid secretion, in some species such as salmonids, the gastrin hormone is not active and histamine stimulates acid secretion. This is the converse of what is observed in mammals, in which the gastric mucosa secretes gastrin after the incoming proteins and then this stimulates the acid secretion and the release of pepsinogen (Jobling, 1995; Lovell, 1988).

Enzymes other than pepsins have been detected in the stomachs of several fish species (Corrêa et al., 2007; De Almeida et al., 2006; Lundstedt et al., 2004). Amylases, lipases, chitinases, and laminarinases can be extracted from the stomachs of several fish, but their efficacy remains little investigated (Chakrabarti et al., 1995; Sabapathy and Teo, 1993). In agastric fish, where there is no HCl production or acidic protease activity, the role of pepsin is played by the alkaline proteases present in the intestine (De Silva and Anderson, 1995).

Fish larvae never present a stomach, which will appear during metamorphosis or, sometimes, later. Certain families, such as cyprinids, are agastric; in other families, the stomach is absent only in some genera or species. Fish that eat coral and ingest large amounts of calcium carbonate, herbivorous fish, or microphagous fish do not have a stomach (Guillaume and Choubert, 2001).

Intestine

The functions of the gut are to complete the digestion started in the stomach and absorb the nutrients, water, and electrolytes, making them available to the metabolism. As a consequence, the walls of the intestines present numerous folds—villi and microvilli—that increase the superficial area for nutrient absorption, allowing greater contact between the cells and nutrients of the lumen. Microvilli are typical structures of absorption tissues and the place where most of the enzymatic digestion of food occurs with the aid of pancreatic enzymes or secretory cells of the intestinal wall (Lovell, 1988).

The intestinal pH ranges from 7.0 to 8.0. The flow of acid chymes from the stomach to the intestine stimulates the production of secretin, which tat reaches the pancreatic tissue through the bloodstream and stimulates the production of bile in the gall bladder and secretion of bicarbonate in the intestine, raising the pH to about 7.0. The release of cholecystokinin (CCK) is stimulated by the presence of amino acids and lipids in the intestine; the CCK stimulates the secretion of several pancreatic enzymes active at a pH between 7.0 and 8.0 and promotes the release of bile into the intestine (Jobling, 1995; Lovell, 1988). The enterocytes are the absorptive cells of the intestinal epithelium, and the products from digestion enter these cells in many different ways: it may be by active or passive transport (e.g., fatty acids and monoacylglycerols) or with or without the presence of a carrier (molecule). Tri and dipeptides, amino acids, and monosaccharides, for instance, are absorbed by means of an ion-bonded, cotransport mechanism (Jobling, 1995). Fat-soluble vitamins are absorbed via micelles while water-soluble substances diffuse through the membranes by means of specific carriers or by passive diffusion. Minerals are absorbed with the help of transporters or via ion channels; oligonucleotides generally bind to amino acids (Guillaume and Choubert, 2001).

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The external anatomical undifferentiation of the fish intestine gives rise to a diversity of terminology in its description. Sections such as the anterior (also cranial or proximal), middle, and posterior (also distal) intestine, duodenum, jejunum, and ileum may have different meanings depending on the author. Segments can be differentiated by the presence of lumen constrictions and, more frequently, by changes in the pattern of villousness (Takashima and Hibiya, 1995). Only histological studies enable recognizing three or four regions and their different functions (Guillaume and Choubert, 2001).

In agastric fish, the proximal or duodenal bowel has a protuberance that may be confused with a true stomach. The function of such a structure is to store food temporarily after feeding, and it differs from the stomach by characteristics such as the pH that is never acidic, the absence of a gastric secretory cell, the absence of peptic activity, and its antero-posterior connection with the biliary tube (Guillaume and Choubert, 2001).

The proximal intestine has a blind bottom diverticulum termed cecum only in fish with stomachs. The number of ceca can range from zero to thousands. Histological studies reveal no striking difference between true intestines and ceca, which thus can be regarded as an extension of the intestinal surface area, thus possessing absorptive functions (Guillaume and Choubert, 2001) and working in association with enzymatic digestion (endopeptidases and exopeptidases of pancreatic origin can actually be detected in the cecum) (Cowey, 1988). The pyloric cecum is embryologically equivalent to the exocrine pancreas and may have an equivalent physiological role in some cases. The structure is, therefore, of great relevance in the digestive process of fish because it can produce a large number of enzymes such as trypsin, chymotrypsin, carboxypeptidases (proteolytic enzymes), amylases, lipases, and chitinase (De Silva and Anderson, 1995). In addition, the pancreatic tissue may also be associated with adipose tissue lining the pyloric ceca or dispersed in the mesentery, as occurs in many teleosts (Kapoor et al., 1975).

The midgut or jejunum can be differentiated from the proximal intestine or duodenum only histologically. Enterocytes have histological adaptations such as many invaginations and large vacuoles, similar to those present in ileum cells of newborn mammals responsible for the absorption and intracellular digestion of protein molecules (Guillaume and Choubert, 2001). As a rule, the distal intestine or ileum is very short. Enterocytes present few microvilli and few characteristics of absorption cells. However, they present many mitochondria, which allow such cells to play a rather important role in osmoregulation by fish through the selective absorption of mineral ions (Guillaume and Choubert, 2001). Finally, the rectum is clearly distinguishable from the anterior sections by the presence of the ileorectal valve, a thicker muscular layer with an abundance of mucous cells (Takashima and Hibiya, 1995).

The length of the intestine varies to a great extent among fish and is related to body length and diet. Ordinarily, carnivores have short intestines while herbivores show long intestines. A study comparing the relative length of the intestine (CRI: intestinal length/total body length) and the feeding habits of 50 fish species of the Characidae and Cyprinidae families reports a gradual increase of the CRI from strict carnivores to strict herbivores (Kapoor et al., 1975). Such differences in bowel length imply important effects on the retention time of the food and, perhaps, on the absorption efficiency of the species. Species with a relatively small intestinal length have increased absorption capacity as a result of mucosal fold amplification. In addition, fish with short intestines, such as carnivores, feature significant nutrient uptake in the posterior intestine, whereas those with long intestines (e.g., herbivores) feature reduced transport of sugar and amino acids at this section (Ferraris and Ahearn, 1984).

Liver and pancreas

The functions of the liver in fish are similar to those of higher vertebrates; it is always a well-developed organ, with copious secretion of bile and a diverse anatomy associated with the taxon. Similar to some birds, some fish present an enlarged liver, which triggers an adipogenic function. Compared to mammals, the fish liver has no lobes at the histological level (Guillaume and Choubert, 2001). The liver secretes emulsifiers that are carried by the bile into the intestine and aid the digestion of lipids (Weatherley and Gill, 1987).

The pancreas of Chondrichthyes and a few Osteichthyes (eels, channel catfish, lungfish, and coelacanths) form a compact organ, the same as for higher vertebrates. The remaining vast majority of species present the pancreas as a diffuse tissue, usually in the form of islets or small glands located along the portal vein that can penetrate neighboring organs, thus forming a "hepatopancreas" (as found in common carp). Enzyme secretions flow through the bile duct into the lumen of the intestine (Guillaume and Choubert, 2001).

Pancreatic secretions include bicarbonate, whose function is to neutralize chyme acid, and zymogens, whose enzymes digest proteins, polysaccharides, lipids, chitin, and nucleotides. This process is aided by the secretion of bile. The pancreas is the most likely source of enzyme production, but its diffuse nature in many teleosts presents a problem in locating the precise source of these secretions. In higher vertebrates, the digestive tract, liver, and pancreas are considered the major endocrine organs. Endocrine secretion of cholecystokinin, gastrin, glucagon, and other oligopeptides has been studied and

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largely reported in fish (Noaillac-Depeyre and Hollande, 1981; Guillaume and Choubert, 2001; Kurokawa et al., 2003; Volkoff, 2006), but their importance and role are still unclear.

Evolution of digestive processes and aspects of nutritional ecology

The food habit

Based upon their feeding habits, fish are grouped into carnivores, omnivores, herbivores, and detritivores. Several species adapt their diet to the local food availability, ranging from bacteria and algae (i.e., plankton) to macrophytes and fish. The food habit may also vary according to the fish's life cycle. A given species may be, while at the juvenile stage, zooplanktophagous, and as an adult, a herbivore, for instance (Barrington, 1957; Chakrabarti et al., 1995). The feeding habit may also vary intra- or interspecifically, depending on the nature of the available food, the feeding site, and/or the ontogenetic stage of the animal.

Fish can also be further grouped into filterers, plancktophagous, benthophagous, and piscivorous (predators). These differences are based not on the feeding habit per se, but on dietary behavior, gastrointestinal tract morphology, and digestive physiology (Chakrabarti et al., 1995; Kapoor et al., 1975). Another possible classification, based on the diversity of food items, is (i) consumers of a mixed diet, euryphagous; (ii) consumers of a limited selection of food types, stenophagous; and (iii) consumers of only one type of food, monophagous (Kapoor et al., 1975).

Diet adaptations

Dietary adaptations can be generically classified as reversible or irreversible (Buddington et al., 1987). Although all adaptations are of a genetic nature, the irreversible adaptations are also termed fixed or genetic while the reversible adaptations are termed biochemical or phenotypic. The mechanisms that activate or control these adaptations differ to a great extent and may or may not be specific. In genetic adaptations to the diet, nonspecific controlling mechanisms are those that, if altered, will affect the absorption of all nutrients; for example the long intestine of the herbivores and the more developed mucosa with the presence of pyloric cecum in carnivores while the modulation of the expression of glucose or amino acid transporters is among the specific adaptive genetic mechanisms. These modifications occur throughout adaptive history. Among the phenotypic or biochemical adaptations to the diet, the mechanisms involved are specific, including different types of regulation of the biomolecules without modifications of the historically defined gene structure. So there is, for instance, adaptive changes to dietary nutrient contents observed through changes in the maximum rate of absorption (V_{max}), the specific enzyme content produced by acinar cells, isozyme production, intermediate action on phenotypic characteristics of biomolecules (K_m and V_{max}), hormonal signaling, etc.

Changes in trophic habitat may lead to intraspecific phenotypic variations with patterns associated with the type of prey or food niche exploited by a given species. In addition, the exploitation of a prey set in an environment can be reduced to a small fraction, exploited by a particular group of individuals only. This trophic polymorphism is usually associated with behavioral, morphological, and physiological adaptive features. Many of these adaptations seem to reflect a phenotypic plasticity, rather than modifications of the genetic structure, an ordinary consequence of a short-term diet specialization. These adaptations may involve changes in the morphology of the mouth and gastrointestinal tract as well as in the digestive and absorption capacities (Beaudoin et al., 1999; Bryan and Larkin, 1972; Buddington et al., 1987; Buddington et al., 1997; Kapoor et al., 1975; Mittelbach et al., 1999). This phenotypic plasticity that enables individuals to modify their alimentary behavior and regulate their digestive functions in the face of the viability of prey or the composition of the diet carries ecological implications. Species capable of making such modifications are able to exploit a wider range of food resources (Carter and Houlihan, 2001). Therefore, information on dietary variations, the feeding habit, and the ecological (feeding) niche is essential to better understand the digestive strategies of different species (Chakrabarti et al., 1995).

Morphological adaptations of the digestive tract in relation to feeding habits include the position of the mouth, the presence or absence of maxillary teeth and/or pharyngeal plaques, variations in the number of branchial rakers, if any, the presence or absence of a stomach, and the extent of the digestive tract, among others. Thus, in addition to interspecific modifications in the morphology of the digestive tract, changes in the digestive enzyme profile and differences in macronutrient absorption capacity may occur (Barrington, 1957; Kapoor et al., 1975). The fish feeding habit has also been related to digestive enzymes. Carnivore fish, for instance, have a higher proteolytic activity, whereas omnivores and herbivores have higher carbohydrase activity (Lovell, 1988). Because of the low carbohydrase activities coupled with potential metabolic deficiencies, carnivorous fish use lipids more efficiently than carbohydrates, both as a nutrient and an energy source (Krogdahl et al., 2005).

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Functional aspects of the digestive system

The digestive processes

The digestion of ingested food and the resulting absorption of nutrients are processes that depend on the presence and availability of appropriate digestive enzymes at correct sites along the gastrointestinal tract. The distribution of digestive enzymes along the gastrointestinal tract of fish has been considered an adaptation to the composition of the diet in nature (see Reimer, 1982). This process should be understood as a consequence of fish life history and the position of this animal group in the course of vertebrate evolution. The fish alimentary canal shows vestiges of the evolutionary stage anterior to that seen in higher vertebrates (with specific enzyme production sites), in which all the sections could produce the main enzymes. However, differences in the patterns of enzymatic zonation in different groups of fish have been described (Chakrabarti et al., 1995). In spite of that, it is accepted that both digestive processes and enzymes, in their mechanism of action and secretion sites, are very similar among fish, mammals, and other higher vertebrates (Kapoor et al., 1975), even among homeotherms (Chesley, 1934; Hidalgo et al., 1999).

Although the digestive enzymes just studied have been shown to be qualitatively similar to those registered in other vertebrates, the digestive processes in fish are not as well known as they are in mammals (Hidalgo et al., 1999). The distribution of digestive enzymes has been described for several Neotropical species, such as the speckled catfish, *Pseudoplatystoma corruscans* (Lundstedt et al., 2004), the tambaqui, *Colossoma macropomum* (Corrêa et al., 2007; De Almeida et al., 2006), the silver catfish, *Rhamdia quelen* (Melo et al., 2006), the pacu, *Piaractus mesopotamicus* (Honorato et al., 2010; Moraes and Bidinotto 2000), the jaraquis, *Semaprochilodus taeniurus* and *Semaprochilodus insignis*, the eartheater, *Geophagus altifrons*, the silver dollar, *Metynnis hypsauchen*, the arawana, *Osteoglossum bicirrhosum*, the yellow tail catfish, *Phractocephalus hemioliopterus*, the peacock bass, *Cichla temensis* and *Cichla monoculus*, (Lopez-Vasquez et al., 2009), the silver catfish, the piapara, *Leporinus obtusidens*, and the wolf fish (trahira) *Hoplias malabaricus* (Gioda et al., 2017). These activities are probably the result of a small mass of diffuse pancreatic tissue throughout the anterior gut and, in adults, to pancreatic infiltrations in the liver (Cataldi et al., 1987).

The secretion of digestive enzymes in fish is also similar to that of higher vertebrates: gastrin, CCK, and other hormones act on the secretion of zymogens and bile following a postprandial pattern. There is an evident adaptation of the enzymes to the pH conditions of the stomach and duodenum. The optimum activity temperature of almost all enzymes varies between 30 and 40°C. However, cold-water fish present particularly active enzymes at low temperatures (Guillaume and Choubert, 2001). As a rule, the distribution and activity of digestive enzymes vary with feeding habit and intestinal morphology (Sabapathy and Teo, 1993; Hidalgo et al., 1999; Moraes and Bidinotto, 2000; Tengjaroenkul et al., 2000). Even so, species with similar feeding habits present differences in the ability to digest food with implications for the formulation of practical diets. The digestive capacity of fish depends, in addition to the level of digestive enzymes, on the time course the nutrients remain under the action of the enzymes.

Digestive enzymes are inductive and, therefore, the activity of carbohydrases is more intense in herbivores while more intense proteolytic activity is recorded in carnivores. Learning the quantity and specificity as well as the optimal enzyme reaction conditions in the digestive system is key to predict the digestive process in the fish as well as the digestibility of new food or feed items. It is also known that digestive-enzymatic activity is also influenced by the age of the fish (Kuz'mina, 1996), the type of food (Hofer and Schiemer, 1981), its physiological state, and the season (Barrington, 1957; Kapoor et al., 1975).

Fish can modulate their digestive profile according to the different sources, types and quantities of dietary nutrients (Corrêa et al., 2007; De Almeida et al., 2006; Honorato et al., 2010; Lundstedt et al., 2004; Moraes and Bidinotto, 2000), an adaptive characteristic favorable to the species' farming and husbandry (Moraes and Bidinotto, 2000). However, this ability seems to vary among species. Carnivores seem to have limited ability to alter their digestive functions and nutrient transport pathways when compared to omnivores (Buddington et al., 1987; Buddington et al., 1997). The digestive enzymatic pattern reflects, in addition to the feeding habit (herbivory, omnivory, or carnivory), the digestive capacity of the fish. Therefore, digestive enzymes have long been investigated as a way to understand the nutritional requirements of fish and the effect of dietary ingredients and nutrients on the activity of these enzymes (Divakaran et al., 1999).

Considering the inductive nature of the digestive enzymes, the phenotypic flexibility of the species may allow not only the adjustments needed to optimize the nutrients of the diet, but also to explore the adaptive capacity of the fish in relation to alternative sources of metabolic energy (Honorato et al., 2010). However, information available on warm-water fish compared to that for cold-water fish is scarce (Melo et al., 2006). There are few studies with tropical species relating the components of the diets with metabolic and digestive aspects. The difficulty of comparing the enzymatic activities among species is based on the diversified protocol used in the several studies, in relation to the tissue sampling as well as the

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nutritional state of the fish, the washing of the lumen content, the time of the enzyme activity test, and the enzymatic assay per se (different modes of homogenization, substrates, reaction pH, and incubation temperature), etc. (Hidalgo et al., 1999).

Characteristics of digestive enzymes

Vertebrate digestive enzymes can be written in one of the following main categories: (i) enzymes secreted by the pancreas and stomach as zymogens, (ii) membrane enzymes bound to microvilli, and (iii) cellular enzymes (Guillaume and Choubert, 2001). The zymogens have specific ionic dependence and pH of action, are activated in the stomach and especially in the intestine, and are never of oral origin. Membrane enzymes are intestinal enzymes that break down fragments of macromolecules filtered by glycocalyx and are active only when bound to a membrane; they work close to the transport systems and ensure absorption by the enterocyte. Cellular enzymes spread out to areas other than the wall (e.g., in lysosomes) and are active in vitro after cell lysis, which makes their role in extracellular digestion somewhat uncertain.

Not all fish have all the digestive enzymes. Agastric fish, for instance, do not have pepsin. In addition, different segments of the fish's digestive tract have characteristic enzymatic activities, either qualitatively or quantitatively. The site of secretion of the enzyme can also vary interspecifically. The stomach of some fish, for instance, may present amylohydrolytic and lipolytic activity while others do not.

Activation of zymogens occurs by breaking off a peptide that covers and hides its active site. In the pepsin molecule, such peptide hydrolysis begins with the action of hydrochloric acid, followed by the hydrolysis of this peptide by pepsin, which is an autocatalytic process. Pancreatic zymogens are initially activated by enterokinase, triggering a chain of events of the trypsin activity on chymotrypsinogen in autocatalytic processes. The active enzymes act successively on the elastase, collagenase, carboxypeptidase A and B, phospholipase, and colipase. With the exception of the phospholipase-colipase pair, these enzymes act on protein macromolecules through endohydrolysis, creating smaller molecules that cannot be absorbed by other enzymes (endo and exopeptidases), except after total hydrolysis (Guillaume and Choubert, 2001).

Classification of digestive enzymes

As a rule, the digestive enzymes are classified according to their catalytic role in the digestion of proteins, carbohydrates, and lipids. Considering the substrate molecular weight and complexity, the digestive process is usually performed by a set of enzymes with complementary functions. Considering this chapter scope, the focus will be centered only on the digestive enzymes of greater relevance according to their insertion in the pertinent literature.

Proteases

Digestion of proteins is carried out by different proteases, with specific actions on the polypeptide chain. Pepsin, trypsin, and chymotrypsin are proteolytic enzymes of great relevance in the gastrointestinal tract of fish (Hsu and Wu, 1979). Products of their reaction, that is, polypeptides or oligopeptides, are broken down by other *endo*- and *exo*-peptidases into the component amino acids, which are ultimately absorbed (Halver and Hardy, 2002). Digestion of proteins usually begins with the action of pepsin (stomach) and is supplemented by alkaline proteases (intestine) such as trypsin and chymotrypsin (Sabapathy and Teo, 1993).

Collagenases and elastases are additional relevant pancreatic proteases, a group of endopeptidases of the serine protease family, i.e., enzymes carrying an essential serine group at the catalytic site. All these proteases are complementary in action, each one acting on different peptide bonds. Depending on the amino acid sequence of the peptide chain, hydrolysis results in different peptides of many sizes that are subsequently hydrolyzed, more than often incompletely, by oligopeptidases (Guillaume and Choubert, 2001). Among the oligopeptidases, the exopeptidases (pancreatic lysosomal enzymes that are not secreted in the inactive form of zymogen) cleave peptide bonds at the amino-terminal side (aminopeptidase) or carboxy-terminal side (carboxypeptidases), and the dipeptidases cleave internal bonds of the other dipeptides.

Pepsin

Pepsin cleaves peptide bonds in the amino-terminal side of the cyclic amino acid residues (tyrosine, phenylalanine, and tryptophan), breaking the polypeptide chains into smaller peptides (Fange and Grove, 1979). This enzyme is responsible for the initial and partial hydrolysis of proteins in the stomach with the aid of secreted hydrochloric acid; the process is completed by a combined action of trypsin and chymotrypsin in the intestine (Sabapathy and Teo, 1993). Pepsin is active

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in acidic pH like that in the gastric portion and can hydrolyze several proteins, except for mucins, spongins, conchiolin, keratin, or low molecular weight peptides (Sumner and Somers, 1947 *apud* Fange and Grove, 1979).

The inactive form of pepsin, the pepsinogen, is activated when in contact with gastric juice (Guillaume and Choubert, 2001). The activity of pepsin in some fish seems little influenced by dietary protein levels and, considering that some species do not present a stomach, their presence is accepted as a nondeterminant factor for protein digestion. However, this concept should be seen with caution because in some fish, pepsin shows an inductive characteristic against different levels of dietary proteins and/or lipids and/or carbohydrates, which suggests its relevance in these species (De Almeida et al., 2006; Honorato et al., 2010; Lundstedt et al., 2004; Melo et al., 2006).

Trypsin and chymotrypsin

Trypsin and chymotrypsin are the major digestive enzymes secreted in the anterior portion of the intestine. Trypsin works specifically in the hydrolysis of peptide bonds in which the carbamyl function is subscribed by lysine and arginine. Chymotrypsin hydrolyzes peptides whose carbamyl function is subsumed by aromatic amino acid residues, such as tyrosine, tryptophan, and phenylalanine. Chymotrypsin also hydrolyzes amides and esters of these aromatic amino acids. When comparing the two enzymes, it is possible to infer that chymotrypsin exhibits a spectrum of much greater proteolytic activity than trypsin (Lundstedt et al., 2004). However, in view of the great importance of proteins in fish nutrition, a number of studies have focused on the role of trypsin as a nutritional indicator. Taken as a key enzyme in the digestion process, it can activate its own precursor form (trypsinogen) as well as several other proteases, including chymotrypsin. In addition to its self-activation, trypsin is also activated by an enterokinase. The secretion of this enzyme in the intestinal lumen is related to the entry of food into this portion of the tract. Chymotrypsin is activated when chymotrypsinogen is secreted into the lumen and contacts trypsin.

Many studies have compared the digestive enzymes in different species of fish (Hidalgo et al., 1999; Hofer and Schiemer, 1981; Hofer, 1982; Hsu and Wu, 1979; Jónás et al., 1983), and although exceptions can be found, most authors consider that proteolytic activity is less dependent on nutritional habits (carnivorous, omnivorous) than is amylohydrolytic activity. However, only comparative studies of proteolytic and amylohydrolytic digestive activities may reveal the ability of fish to use carbohydrates and the many sources of proteins.

Lipases

Digestion of lipids occurs in the stomach, cecum, and intestine through the action of many lipases and colipases. Dietary lipids, mainly triacylglycerols, are hydrolyzed by nonspecific lipases into free fatty acids and glycerol (De Silva and Anderson, 1995), which are absorbed and used for biosynthetic processes of several cellular components or catabolized for free energy liberation.

Several types of lipases have been reported in the digestive tract of juvenile and adult fish (Izquierdo et al., 2000). Even if the teleost pancreas is diffuse, it is assumed that the digestive lipases of fish are similar in activity to the major mammalian forms. Lipases occur in the stomach of some fish but, for most species, the primary site of lipid hydrolysis appears to be the anterior portion of the intestine and pyloric cecum, when present. However, lipid digestion continues in the other segments of the intestine, especially in fish that are strictly carnivores, which present a short digestive tract (Ferraris and Ahearn, 1984). The distribution patterns of lipolytic activity across the various segments of the gastrointestinal tract of fish, either juveniles or adults, seem to diverge between species (Chakrabarti et al., 1995; Corrêa et al., 2007; De Almeida et al., 2006; Lundstedt et al., 2004; Melo et al., 2006, Santigosa et al., 2008; Lin and Luo, 2011; Silva et al., 2010).

Lipase acts on lipid droplets that are suspended in the aqueous phase, aided by bile salts and colipases in elasmobranchs (Bacha et al., 2011; Smichi et al., 2012). Colipase was not found in several teleosts studied (Sæle et al., 2010; Smichi et al., 2012, 2017). Colipase aids the lipase anchoring on the lipid molecule because it has an affinity for both molecules. After the anchoring, the colipase discovers the lipase site by completely activating this enzyme (Guillaume and Choubert, 2001). The structure of lipases is very similar to that of serine proteases, and along with amylase, these are the only pancreatic enzymes secreted directly in active form. However, they become significantly active in the presence of colipase, secreted in the same tissue. Unlike enzymes that act in an aqueous environment, lipase acts on the interface of lipid beads suspended in the aqueous phase. The presence of bile salts, while increasing this interface by decreasing the surface tension, gives the "positioning" of the lipase in the lipid droplets. Colipase has a double affinity and thus allows the formation of lipid-lipase-colipase bead complexes, where lipase is to some extent anchored to lipids (Guillaume and Choubert, 2001). Additional studies have shown that once the lipase is anchored to the colipase, it discloses its catalytic site (Bacha et al., 2011; Smichi et al., 2012). The considerable activation of lipase in elasmobranchs thus comes from two distinct phenomena.

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Fish lipases differ from those of higher vertebrates because they show a similar affinity for triacylglycerols with different fatty acids and are not especially active in the external (α) position. There are few studies on other lipolytic enzymes in fish, but esterase and phospholipase activities have already been reported (Guillaume and Choubert, 2001). It is difficult to state whether these enzymes are secreted by the stomach or pancreas because lipases generally tend to be nonspecific.

Carbohydrases

Among the major carbohydrases stands amylase, given its presence and distribution in the organisms. It is an $\alpha_{(1\rightarrow4)}$-glycosidase that hydrolyzes glucosyl-$\alpha_{(1\rightarrow4)}$ bonds of glycosyl glucose into linear fragments of amylopectin, branched amylose, or glycogen fragments. However, the amylase hydrolyzes $\alpha_{(1\rightarrow6)}$ bonds that form the amylose and glycogen branches. This enzyme has been reported in all fish sampled and studied, including deep-sea carnivores that never find starch in their food. By externally attacking poly-glucosyl-glucose of linear chains and variable length, the amylase hydrolyzes them into maltoses from their branching points $\alpha_{(1\rightarrow6)}$ as well as the short chains of dextrins.

There are other glycosidases reported in fish and they usually have low activity. Cellulase ($\beta_{(1\rightarrow4)}$ glycosidase) is usually detected but it is also usually involved in exogenous enzyme activities of bacterial origin. The presence of laminarinase ($\beta_{(1\rightarrow3)}$ glycosidase) has been shown in fish that consume algae.

Amylase

Glucose polysaccharide digestion is carried out by a pool of enzymes, the most important being amylase, and it produces a variety of oligosaccharides as reaction products (Lovell, 1988). The digestibility of polysaccharides is low for most fish, and it is directly related to their chemical nature and the digestive capacity of each species. Freshwater omnivorous tropical fish can digest and metabolize carbohydrates relatively well in comparison to carnivores, especially those from temperate climates such as salmonids.

The digestive α-amylase can be spotted throughout the gastrointestinal tract of many fish species (Chakrabarti et al., 1995; Corrêa et al., 2007; Fagbenro et al., 2000, Tengjaroenkul et al., 2000). Amylase is secreted by salivary and pancreatic cells in mammals, but in fish, the only source of α-amylase appears to be the exocrine pancreas (Krogdahl et al., 2005). Along with other pancreatic enzymes, amylase activity is detected within the intestinal lumen, in the chyme, as well as bound to the mucosal cell membrane. This enzyme presents differing characteristics between species in terms of optimum pH and thermal stability (Krogdahl et al., 2005). In addition to the common features, fish amylases also show differences, for example, in the dependence and concentration of ions.

The activity of amylase in tissues and intestinal contents varies among species and appears to be higher in herbivorous and omnivorous fish than in carnivores (Chakrabarti et al., 1995; Moraes and Bidinotto, 2000; Sabapathy and Teo, 1993; Krogdahl et al., 2005). In the carnivore trout, for instance, α-amylase levels are very low (Hidalgo et al., 1999). The ability to adapt amylase secretion to dietary carbohydrate levels and feed intake should be restricted to omnivorous and herbivorous fish (Krogdahl et al., 2005). The increase of amylase production may occur in response to the presence of carbohydrates, or products of their hydrolysis, in the gastrointestinal tract. Glucose can influence directly its pancreatic-tissue production, or indirectly by stimulating the pancreas insulin release (Jobling, 1994). According to some authors, the production of α-amylase is independent of diet. However, other studies have shown that the increase of dietary starch is reflected in its activity (Corrêa et al., 2007; Moraes and Bidinotto, 2000), thus unveiling the ability of this enzyme to be modulated in some fish. The ability to adapt amylase secretion to dietary carbohydrate contents and feed intake seems restricted to omnivorous and herbivorous fish. In addition, there are amylohydrolytic, endogenous inhibitors present in wheat and other grains, possibly working in a species-specific way (Krogdahl et al., 2005). The digestibility of the polysaccharides is directly related to their chemical nature and the digestive capacity of each species (Bergot, 1993). Tropical freshwater fish can take advantage of dietary carbohydrates relatively well when compared to marine fish, particularly salmonids (Lundstedt et al., 2004).

Chitinase

Chitinase, an enzyme driving the hydrolysis of chitin, is present in the digestive system of many species regardless of their feeding habits, with different optimal pH and tissue distribution. Chitin, a linear homopolymer of *N*-acetylglucosamine (NAG) units linked by $\beta_{(1\rightarrow4)}$-glucosidic bonds, is a common component of some bacteria, bacterial walls, and lower plants, and it is the main component of the arthropod exoskeleton (Krogdahl et al., 2005). It is particularly relevant for arthropod consumers, whereas chitinolytic activity is found in the chyme of the entire gastrointestinal tract and, similarly to amylase,

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also in the wall homogenates of the digestive tract. Generally, the highest concentrations are located in the stomach and pyloric cecum, indicating that either these structures or the diets are the main sources of the enzyme. The role of intestinal chitinase in the digestion of carbohydrates and its nutritional value remain challenged (Krogdahl et al., 2005).

Cellulase

Cellulase activity has been observed in many fish species, indicating their ability to utilize cellulose and similar fibrous carbohydrates (Chakrabarti et al., 1995). There are still doubts about its origin: endogenous or microbial.

Disaccharidases

Gastrointestinal homogenates from fish mucosa are able to hydrolyze several low-molecular-weight saccharides, including the disaccharides maltose (glucopyranosyl $\alpha_{(1\rightarrow4)}$ glucopyranose), sucrose (glucopyranosyl $\alpha_{(1\rightarrow4)}$ fructofuranose), and trehalose (glucopyranosyl $\alpha_{(1\rightarrow1)}$ glucopyranose). These enzymes are detected at the brush border of enterocytes and can also be found in the lumen content (Fagbenro et al., 2000), probably originating from mucous desquamation. However, because these enzymes are active in chyme, they must also hydrolyze the substrates present therein (Krogdahl et al., 2005).

The disaccharidases occur in the mucosa of ceca and in the middle and posterior segments of herbivorous, omnivorous, and carnivorous fish intestines (Krogdahl and Sundby, 1999; Ugolev and Kuz'mina, 1994). Their activity varies to a great extent among species and is higher in herbivorous than carnivorous fish (Ugolev and Kuz'mina, 1994). It is also smaller in distal segments than in the middle and pyloric regions. Intestinal segments tend to hydrolyze maltose more than other disaccharides (Krogdahl and Sundby, 1999; Ugolev and Kuz'mina, 1994). Disaccharidase activities differ widely among species and are higher in herbivorous than in carnivorous fish (Ugolev and Kuz'mina, 1994).

Accessory enzymes of the digestive process

Among the accessory enzymes of the digestive process stands the leucine-alanine peptidase, an intestinal lysosomal enzyme (Guillaume and Choubert, 2001). Esterases are endocellular enzymes that act after molecules undergo endocytosis (Guillaume and Choubert, 2001). Aminopeptidases are exopeptidases that act at the amino-terminal end of low molecular weight peptides formed from the endopeptidases action. Laminarinases, also termed $\beta_{(1\rightarrow3)}$ glycosidase, are present in algae-filtering fish (Guillaume and Choubert, 2001). The esterases, which are often found in histoenzimology, are endocellular enzymes that work after the particles undergo endocytosis. The levels of activity of these enzymes are much higher in larval than in juvenile fish. Some intestinal enzymes facilitate the nutrient breakdown and transport. For example, while alkaline phosphatase is involved in the absorption and transport of lipids and carbohydrates, glutamyl transferase plays a role in the transport of amino acids and oligopeptides through the intestinal wall (Fraisse et al., 1981).

Digestion, digestive transit, and absorption

The utilization of dietary nutrients depends on several (digestive) factors that, together, are commonly known as digestibility. In addition to these factors, previously discussed, the speed with which the food bolus passes through the digestive tract, termed digestive transit time, is another important factor to achieve the climax of the nutrient utilization process, the absorption process. The digestive transit time reflects the motility of the digestive system, which is also linked to the origin (kind) and proportion of dietary ingredients (Honorato et al., 2010). The food bolus can be more or less retained and exposed to the digestive factors and then undergo the absorption process. Thus, the transit time directly influences the biological efficiency of the nutrients (NRC, 2011).

Fish that have stomachs can retain the ingested food at this organ for relatively long time intervals. At temperatures below 10°C, this time interval can reach tens of hours (Guillaume and Choubert, 2001). The stomach contribution to digestion is remarkable when the fish, for example, swallow the whole prey. Its digestion process begins from the prey outer tegument and occurs as a result of intense proteolytic activity (in particular, elastase activity) along with the chitinase activity.

The evacuation of gastric contents is a multifactorial mechanism as it depends on the species, the size of the fish, the type and amount of food ingested, and the water temperature. It is generally accepted that evacuation kinetics can be expressed by a differential equation influenced by the stuffing of the stomach, that is, the faster the filling, the faster the emptying. Moreover, from a certain level of emptying, the organism no longer has the sensation of fullness and the appetite returns. That all leads herbivorous fish to have a shorter transit time than carnivorous fish (Guillaume and Choubert, 2001).

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Secretion of enzymes, transit time, and absorption are characteristics of the digestive process that, together, determine the overall efficiency of the whole process, which depends on the regulation of the interrelationship of these three factors, mainly the absorption mechanisms. However, studies on the interaction between these factors remain scarce (García-Meilán et al., 2014; Canada et al., 2017). Studies regarding the interaction between these four aspects have focused on the significant influence of fish age or size, nutritional balance, or temperature in the interaction of those factors. Except under pathological stages, the interrelation between these factors seems to be under remarkable regulation. For example, a drop in temperature decreases the enzymatic activity and the rates of absorption, slows the gastrointestinal transit, and decreases food intake. However, at least in eurythermic fish, these factors only affect the general balance (Guillaume and Choubert, 2001). An increase in dietary protein as well as carbohydrate and lipid content leads to a rapid stimulation of trypsin, amylase, and lipase secretion, respectively. This regulation is neither instantaneous nor accurate, but the regulation of absorption mechanisms (as found in higher vertebrates) is generally sufficient to keep the overall effectiveness of the process practically unchanged after a period of adaptation. The fitting period spent on these adjustments is, in most cases, 1 day. The final digestive balance usually remains unchanged, showing the existence of a balance between ingestion, enzymatic secretion (digestion itself), transit time, and absorption (Guillaume and Choubert, 2001).

Final considerations

Since the second half of the twentieth century, aquaculture has been recognized globally as a viable and profitable investment, and it is even a crucial activity in several regions, expanding rapidly in the world agricultural segment. The increase of fish consumption occurs in a world scenario in which society demands diversification of the protein sources, shows an increase of the average per capita income, and a preference for fish over other animal protein sources, whether for personal, cultural, or health reasons. These factors, when interrelated, accelerated the growth of the fish industry. About 60% of the world's landings of seafood will be represented by aquaculture by the year 2030. The Neotropical region amasses an incomparable number of fish species of the most varied shapes and sizes, many of them promising for fish farming, as actually long reported by Saint-Paul (1986), for instance. However, in spite of recent developments in farming and husbandry techniques, many farmed fish remain improperly fed due to the unsatisfactory understanding of their nutritional requirements and feeding behavior.

When properly managed, fish production in confined systems presents higher yields. However, the production requires a rational increase of food supply either through fertilization or the supply of nutrients in balanced diets (Pillay and Kutty, 2005). It is essential to be aware that fish productivity is a consequence of the animal biological responses to the environmental or farming conditions. The knowledge of the biological plasticity of farmed fish is of great importance to the reconciliation of animal productivity, its welfare, environmental sustainability, and the maximization of the dietary nutrients (Honorato et al., 2010).

Feeding and nutrition may account for nearly 70% of production costs and a significant reduction in these costs can be achieved by using high-quality ingredients, effective feed processing techniques, and refined feeding strategies. Thus, the importance of studies on fish feeding and nutrition, focusing on macronutrient utilization, digestion, and yield, is at an all-time high. The optimization of confinement production conditions of economically viable fish species not only will increase profitability of farming systems, but will also reduce environmental impact (Halver and Hardy, 2002). These studies are based on the specific ability of the fish to use the food, on its metabolic response to the nutritional variation of the diet (Reimer, 1982), and in the influence of factors that act on the utilization of these nutrients. Among these factors, the interrelationship between the enzymatic activities of the digestive processes and the adaptive metabolic profile has shown satisfactory results (Corrêa et al., 2007; De Almeida et al., 2006; Honorato et al., 2010; Lundstedt et al., 2004; Melo et al., 2006; Moraes and Bidinotto, 2000). The role of nutrition in aquaculture activity is clearly recognized, and efforts shall continue being made to understand the interrelationships between nutrient levels and digestibility, absorption, growth, and the health of fish produced in fish farming (Halver and Hardy, 2002).

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Chapter 12

Osmotic and ionic regulation

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Introduction

Freshwaters vary broadly in regard to ions and organic compound contents. The contents of ions in freshwater depend heavily on the water body substrate. If such a substrate is made up of insoluble rocks, such as granite, the water will have a limited content of divalent ions, which means that it will be termed soft water. A substrate made up of limestone, for instance, will release large amounts of Ca^{2+} and Mg^{2+} ions to the water, which will be termed hard water (Table 12.1).

The water of some Neotropical rivers, such as the Negro River of the Amazonian region, does have a low ion content (Table 12.1), a pH of 5.0–6.0, and a large amount of dissolved organic matter (Aucour et al., 2003; Mortatti and Probst, 2003), which taints the water a blackish color. The waters of rivers having large contents of suspended sediments, neutral pH, and comparatively rich ion content, such as the Solimões River (Aucour et al., 2003; Tardy et al., 2005), will be light brown or whitish in color, thus termed white waters (Table 12.1). Both are soft waters, but the ionic contents of the Solimões River are 5–10 times larger than those of the Negro river. In the flooding season or where black and white water rivers merge, for example, the Negro and Solimões Rivers "meeting of waters" to form the Amazon River, many fish migrate from one type of water to another (Araújo-Lima and Goulding, 1997; Barthem and Goulding, 1997). In the dry season, fish living in black waters may have to cope with acidic waters (pH 3.5–4.0) pouring in from the small streams (igarapés) (Matsuo and Val, 2003). In addition to the black and white waters, the Amazon region also encompasses the so-called clear water rivers, such as the Tapajós River, exhibiting neutral pH and Ca^{2+} concentrations twice as high as black waters (Konhauser et al., 1994). Other Neotropical rivers and lakes may have soft and ion-poor waters, with the exceptions of specific sites such as some lakes of the Brazilian Pantanal (Table 12.1) and some Peruvian rivers (Wasiw and Yépez, 2015). Some salt lakes (salinity ranging from 1.35 to 26.1 ppt) are found in the Chaco-Pampean plain and the Patagonian province (Argentina) (Puntoriero et al., 2015).

Because fish body fluids are more concentrated than freshwaters, that is, fish are hyperosmotic in relation to the aquatic environment, efficient osmoregulation pathways are an important feature of the freshwater fish metabolism (Tables 12.1 and 12.2). Therefore, freshwater fish face elementary problems such as excessive water uptake by osmosis and loss of ions by diffusion, so they have to avoid ion loss (or having efficient mechanisms to capture ions from the environment) and eliminate excess water (or prevent its entry into the body) on a permanent basis (Fig. 12.1). This chapter aims at addressing the main organs and mechanisms of osmoregulation in freshwater fish, focusing on Neotropical species while also analyzing the relationship of some physicochemical parameters of the water with the osmoregulatory mechanisms of these fish.

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TABLE 12.1 Ion concentrations (in mmol L^{-1}) in several freshwater environments from the Neotropical region

	Na^+	K^+	Cl^-	Ca^{2+}	Mg^{2+}	HCO_3^-	pH
Negro river (Brazil)[a,b]	0.039	0.006	0.014	0.007	0.004	0.018	4.6–5.8
Solimões river (Brazil)[c]	0.135	0.026	0.117	0.248	–	–	7.0
Pantanal (Brazil)[d]	11.1	3.0	–	0.23	0.04	–	9.33
Vacacaí river (Brazil)[e]	0.198	0.036	–	0.055	0.078	–	5.0–7.9
Lake Laguna Negra (Chile)[f]	0.130	0.078	0.192	0.502	0.079	–	7.1
Lake El Espinal (Argentina)[g]	1.626	0.579	0.806	1.520	0.235	5.079	7.7
Ostional River (Nicaragua)[h]	1.01	0.02	0.68	9.48	1.04	6.60	7.5
Lake Zapotlán (Mexico)[i]	0.243	0.049	0.08	0.297	0.346	–	–

[a] *Mortatti and Probst (2003).*
[b] *Küchler et al. (2000).*
[c] *Baldisserotto et al. (2008a).*
[d] *Galvão et al. (2003).*
[e] *Kochhann et al. (2013).*
[f] *Echeverria-Veja et al. (2018).*
[g] *Babini et al. (2016).*
[h] *Calderon et al. (2014).*
[i] *Farrick and Branfireun (2015).*

Organs involved in osmoregulation

Epithelia

As a rule, the secretion of mucus to reduce the gas and ion exchanges between the animal and the water is rather abundant in freshwater fish (Glover et al., 2013). In some species that may present complementary air breathing mechanisms (Johansen, 1968), such as the marbled swamp eel (*Synbranchus marmoratus*), the body surface may also contribute with the absorption of Na^+. Records of low urine flow rate (1.8 mL kg^{-1} h^{-1}) and low rate of urinary sodium excretion (17.9 μmol kg^{-1} h^{-1}), especially when combined with extra-renal Na^+ loss (3.9 μmol kg^{-1} h^{-1}), indicate that the marbled swamp eel has a very low Na^+ turnover rate. In addition, experiments that divided the Na^+ flows into the proximal head (gills and skin) and distal part of the body (skin and urinary system) showed that about 75% of the Na^+ influx occurs through the body surface (Stiffler et al., 1986). In some Teleosts, ionocytes (chloride cells, PNA+, or mitochondria-rich cells) are recorded on the epithelial layer of the head, buccal cavity, and opercular membrane. These ionocytes are related especially to the uptake of Ca^{2+}, but also to Na^+ and Cl^- uptake (Marshall and Bellamy, 2010). The mechanisms of absorption of these by the ionocytes are described ahead.

Gills

Although water uptake and ion loss by diffusion can occur in the gills of freshwater Teleosts, the permeability of junctions between the gill cells of such fish is rather poor as a result of the presence of occludin and claudin proteins (Chasiotis et al., 2012). Ca^{2+}-linked, negatively charged amino acid residues are also detected at these junctions. In addition, the mucus produced by mucosal cells, located in the gills, carries electrically charged radicals. That attracts ions to the cell surface, resulting in an ionic gradient of the gill membrane to the water, reducing the loss of ions by diffusion.

The gills of freshwater Teleosts present both ionocytes and pavement cells (PNA −) (Evans, 2011; Shahsavarani et al., 2006), but about 90% of the cells that make contact with the water are pavement cells (Wilson and Laurent, 2002). Ionocytes are usually placed where the gill filaments intersect the lamellae, but fish dwelling in soft water may register proliferation of the ionocytes in the lamellae. The apical surface of ionocytes varies according to species and environmental conditions. The apical membrane may have microvilli, be smooth or have spongy surface. In some species, ionocytes are buried in the epithelium and the apical surface is almost completely covered by pavement cells. Several types of ionocytes were identified in freshwater teleosts, five of them in zebrafish, *Danio rerio* (Guh et al., 2015). An Na^+/K^+ ATPase may be found in the basolateral membrane, eliciting an Na^+-favorable gradient; the apical membrane may carry an H^+ ATPase

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TABLE 12.2 Ion concentrations (in mmol L^{-1}) in plasma and bile of some freshwater fish from the Neotropical region

Species	Plasma	Bile	Plasma	Bile	Plasma	Bile	Plasma	Bile	Plasma	Bile
	Na^+		K^+		Cl^-		Ca^{2+}		Mg^{2+}	
Hoplias malabaricus[a]	180.4	–	7.0	–	80.0	–	5.0	–	1.8	–
Hoplias malabaricus[b]	148.8	152.4	5.5	5.5	71.7	13.3	2.2	9.8	1.3	4.3
Hoplosternum littorale[a]	176.8	–	9.0	–	98.0	–	6.0	–	1.85	–
Rhamdia quelen[a]	175.9	–	5.0	–	95.0	–	5.0	–	2.1	–
Rhamdia quelen[c]	160.1	180.1	3.5	7.0	110.1	20.0	–	–	–	–
Synbranchus marmoratus[d]	138.0	172.1	2.8	7.5	62.6	22.9	2.85	8.3	1.65	2.55
Prochilodus affinis[d,e]	116.6	164.0	5.5	7.9	104.0	34.4	2.45	11.2	0.95	2.55
Prochilodus marggravii[d,e]	124.1	173.6	4.3	7.8	117.9	28.9	2.5	10.7	1.1	2.25
Prochilodus scrofa[d]	–	210.9	–	6.4	–	11.1	–	9.1	–	1.8
Arapaima gigas[f]	–	380.0	–	21.0	–	55.0	–	4.0	–	–
Geophagus altifrons[f]	–	210.0	–	23.0	–	55.0	–	6.0	–	–
Cichla temensis[f]	–	380.0	–	23.0	–	32.0	–	6.0	–	–
Cichla monoculus[f]	–	250.0	–	25.0	–	85.0	–	5.5	–	–
Paratrygon aiereba[g]	138.9 (NR)	–	4.8 (NR)	–	146.2 (NR)	–	2.6 (NR)	–	–	–
	156.6 (AR)	–	5.7 (AR)	–	177.5 (AR)	–	2.9 (AR)	–	–	–
Potamotrygon sp.[h]	178.2	–	–	–	146.2	–	–	–	–	–

[a]*Becker et al. (2006).*
[b]*Baldisserotto and Mimura (1997).*
[c]*Becker et al. (2009).*
[d]*Baldisserotto et al. (1990).*
[e]*Mimura and Baldisserotto (1988).*
[f]*Baldisserotto et al. (2004b).*
[g]*Duncan et al. (2009)—specimens collected in the Negro river (NR) and in the Amazon river (AR).*
[h]*Wood et al. (2002).*

(V-ATPase) that eliminates H^+ and increases the electric gradient to facilitate Na^+ entry into the cell through a Na^+ channel located in the apical membrane (Duarte et al., 2013; Evans et al., 2005; Hwang and Lee, 2007).

In waters with pH above 7.6 or low Na^+ concentration, part of the Na^+ uptake seems to result from the Na^+/H^+ antiporter in the apical membrane, which allows Na^+ absorption and H^+ elimination, aiding the acid-base equilibrium. Cl^- is absorbed through the Cl^-/HCO_3^- antiporter, also located in the apical membrane. Both the H^+ and HCO_3^- used in these ionic

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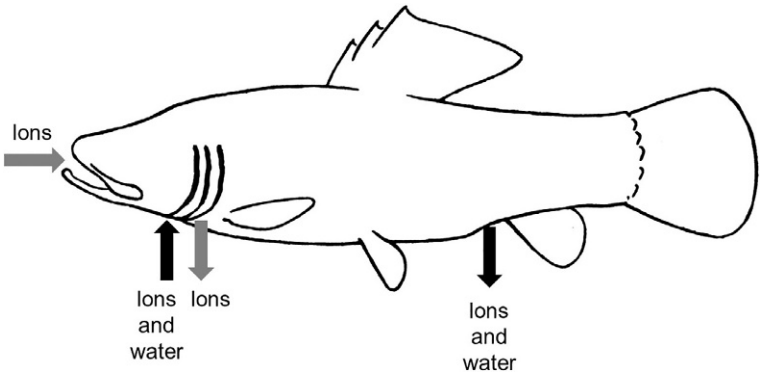

FIG. 12.1 General representation of ion and water fluxes in fish adapted to freshwater. *Black arrows* indicate inflow or absorption; *gray arrows* indicate efflux or excretion.

exchanges come from the dissociation of the carbonic acid formed by the combination of the CO_2 with the water driven by the carbonic anhydrase, that is, there can also be an integration between the absorption of ions and the elimination of the CO_2 resulting from the respiration (Evans et al., 2005). The presence of transporters can be also demonstrated in Neotropical species (Souza-Bastos et al., 2015). The withdrawal of the Cl^- in the basolateral membrane seems to occur through a cystic fibrosis transmembrane conductance regulator [CFTR]-type Cl^- channel (Hwang and Lee, 2007). The presence of an Na^+/HCO_3 antiport can also be detected in the basolateral membrane of the pavement cells, which would transport Na^+ from the intracellular medium to the extracellular medium and cause an intracellular acidification because of the withdrawal of HCO_3^-. This leads to the opening of Na^+ in the apical membrane, increasing its absorption (Parks et al., 2007) (Fig. 12.2). Because ion uptake in the gills has been studied in only a few species, it is still unclear whether the different transport systems described result from exposure to different environments or are species-specific. It is possible that the Cl^-/HCO_3^- antiporter also helps in the elimination of excess HCO_3^- that enters the blood after a meal, aiding in the acid-basic balance of the fish (Cooper and Wilson, 2008).

The uptake of Ca^{2+} in the gills and opercular membrane of freshwater Teleosts depends on a Ca^{2+} ATPase (mainly) and the Ca^{2+}/Na^+ antiporter present in the ionocytes. Both systems transport Ca^{2+} from within the cells to the plasma, reducing

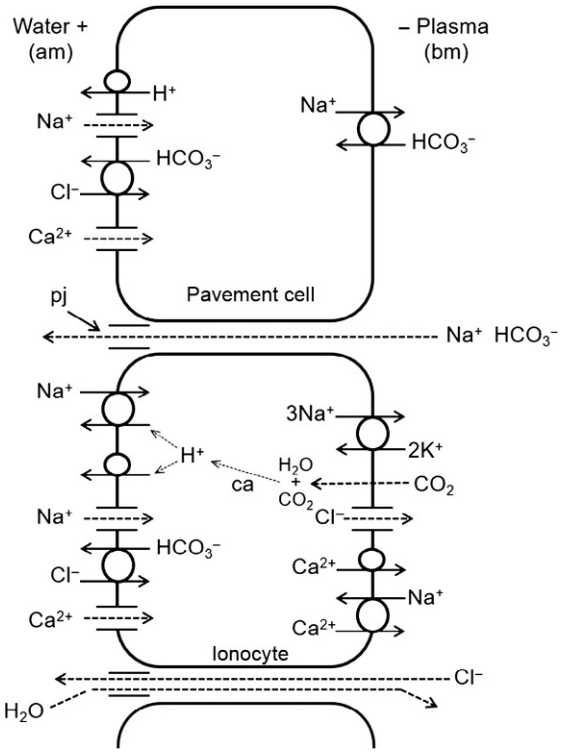

FIG. 12.2 Ion fluxes in ionocytes and pavement cells of gills of freshwater Teleosts. *Circles* represent transport systems; *dotted lines* diffusion. *am*, apical membrane; *bm*, basolateral membrane; *ca*, carbonic anhydrase; *pj*, protein junctions linking the gill cells.

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the intracellular concentration of this ion, which facilitates its entry from the medium into the ionocyte through a Ca^{2+} channel (Liao et al., 2007; Shahsavarani et al., 2006), also found in the pavement cells, suggesting that Ca^{2+} uptake may not be restricted to ionocytes (Shahsavarani et al., 2006) (Fig. 12.2).

The structure of the gills of the water-breathing larvae of pirarucu, *Arapaima gigas* (Osteoglossomorpha: Arapaimidae) is similar to that of most teleost fish, for example, Characins. However, the morphology of the gills changes with the growth of fish, with the occurrence of intense cell proliferation in the filaments and lamellae. This increases epithelial thickness and decreases interlamellar distance, accompanied by intense ionocyte proliferation in the lamellar epithelium. All these morphological changes in the gills increased the water-blood diffusion distance, and the dependence on air-breathing when fish grows, while the proliferation of the ionocytes may increase ion regulation (Ramos et al., 2013).

Gastrointestinal tract

Absorption of Na^+ in the marbled swamp eel and the Characin curimbatá, *Prochilodus scrofa*, may occur in the stomach (Baldisserotto et al., 1993; Baldisserotto and Mimura, 1995) while other ions are absorbed in the intestine or pyloric cecum when present (Baldisserotto et al., 1993, 1996b, 2006; Baldisserotto and Mimura, 1994, 1995). The source of ions for absorption in the gastrointestinal tract is ingested food (Becker et al., 2006, 2012), ions released by gastric secretion (Cl^-) (Becker et al., 2006, 2012; Bucking and Wood, 2006b), and biliary and pancreatic secretions (Table 12.2) (Baldisserotto et al., 1990, 2004b; Baldisserotto and Mimura, 1997; Becker et al., 2009). Water intake by freshwater fish is low (Flik et al., 1986), but the water secreted in the stomach and anterior intestine is reabsorbed in the final portions of the intestine (Bucking and Wood, 2006a). In rainbow trout, 80%–90% of the K^+ and Cl^- originated from the feed are absorbed while the absorption of Na^+ resulting from the feed is very small, that is, the intestine reabsorbs an amount corresponding to all the endogenous Na^+ secreted in the lumen, but there is no net gain of Na^+ from feed and food (Bucking and Wood, 2006b).

The entry of Na^+, Cl^-, and K^+ into enterocytes of fish occurs through the $Na^+/2Cl^-/K^+$ symporter in the apical membrane (Baldisserotto and Mimura, 1994; Marvão et al., 1994), but apparently in the later portions of the intestine, the Na^+/Cl^- symport also drives the absorption of Na^+ and Cl^- (Cutler and Cramb, 2008). The passage of these ions from the intra- to the extracellular medium through the basolateral membrane is done through the Na^+/K^+ ATPase and the K^+/Cl^- symporter. Other transport systems appear to be more related to the acid-base balance of the intracellular medium (Baldisserotto and Mimura, 1994; Marvão et al., 1994). There is absorption of Ca^{2+} in the intestine of rainbow trout, but the main system for transporting these ions from the enterocyte to the circulatory system through the basolateral membrane is the Ca^{2+}/Na^+ antiporter while the Ca^{2+} ATPase plays a secondary role. In the apical membrane, Ca^{2+} enters the enterocyte through an L-type Ca^{2+} channel and probably through a Ca^{2+} channel or Ca^{2+} channel mediated by a purinoreceptor (Baldisserotto et al., 2006) (Fig. 12.3).

Gut sac experiments in vitro using solutions with similar concentrations of both the serous and mucosal sides demonstrated net water absorption and Ca^{2+} secretion in the middle and posterior intestines, respectively, and absorption of Na^+ and K^+ in the mid and posterior gut, respectively, with no significant uptake of Mg^{2+} (Cl^- uptake was not analyzed) of wolf fish (*Hoplias malabaricus*) (Baldisserotto et al., 1996b). These findings help explain the decrease in Na^+ levels and the fourfold increase in Ca^{2+} levels in the fluid phase from the pyloric ceca to the posterior gut of this species (Becker et al., 2006) (see Table 12.3). In addition, water uptake may possibly explain the increase in Cl^- levels in the fluid phase of the middle and posterior intestines whenever the intestinal absorption of Cl^- is reduced or absent. However, apparently there are not significant changes in the levels of K^+ and Mg^{2+} in the intestinal contents (Becker et al., 2006) (see Table 12.3 and Fig. 12.4). A comparative study of in vitro gut sac preparations demonstrated intestinal absorption of Na^+ and Cl^- in the Characins black piranha (*Serrasalmus rhombeus*), tambaqui (*Colossoma macropomum*), and pacu (*Myleus lobatus*) (Pelster et al., 2015).

The absorption of ions in the in vitro digestive tract of the marbled swamp eel depends on the pH of the medium. At pH 8.0, that is, close to the expected measure in the intestinal contents, there is net Na^+ and Ca^{2+} absorption and water secretion in the anterior intestine (Baldisserotto et al., 1993). Comparable in vitro experiments showed that the pyloric ceca of curimbatá secretes water, K^+, Ca^{2+}, and Mg^{2+}, but the middle and posterior intestines absorb Na^+, Cl^-, and water, and secrete Ca^{2+} and Mg^{2+}; K^+ is secreted only in the posterior intestine. Na^+ and water are absorbed in the posterior intestine while K^+ and Ca^{2+} are secreted. It is therefore evident that the digestive tract plays an active role in the osmoregulatory processes of this species, and that the stomach and the mid and posterior intestines are the main sites of Na^+ and Cl^- absorption.

The foregut (or pyloric ceca) of Teleosts receives bile, which has ions at a higher concentration than plasma, with the exception of Cl^- (Baldisserotto et al., 1990, 1996a, 2004b; Baldisserotto and Mimura, 1997; Becker et al., 2009) (Table 12.2). The ion levels in the vesicular bile may vary according to the phase of the feeding (Baldisserotto and

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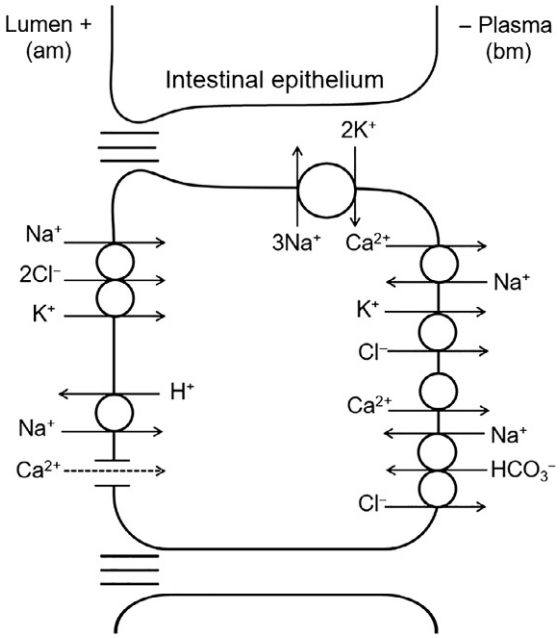

FIG. 12.3 Ion transport systems in the guts of freshwater Teleosts. *Circles* represent transport systems; *dotted lines* diffusion.

TABLE 12.3 Ion levels (in mmol L^{-1}) in different segments of the gastrointestinal tract of some teleost fish.

	Segments of the gastrointestinal tract			
Species	**Stomach**	**Anterior intestine**	**Middle intestine**	**Posterior intestine**
Na^+				
Hoplias malabaricus	32.0	180.0	80.0	62.0
Rhamdia quelen	28.0	68.0	70.0	82.0
Hoplosternum littorale	30.0	75.0	–	65.0
K^+				
Hoplias malabaricus	9.0	10.0	15.0	13.0
Rhamdia quelen	25.0	40.0	15.0	12.0
Hoplosternum littorale	32.0	18.0	–	9.0
Cl^-				
Hoplias malabaricus	40.0	45.0	76.0	65.0
Rhamdia quelen	170.0	110.0	85.0	55.0
Hoplosternum littorale	70.0	50.0	–	48.0
Ca^{2+}				
Hoplias malabaricus	8.0	9.0	32.0	38.0
Rhamdia quelen	35.0	8.0	9.0	17.0
Hoplosternum littorale	5.0	4.0	–	4.5
Mg^{2+}				
Hoplias malabaricus	5.0	9.5	11.0	10.0
Rhamdia quelen	2.8	5.0	1.8	5.3
Hoplosternum littorale	2.3	4.0	–	1.8

Data from Becker, A.G., Gonçalves, J.F., Garcia, L.O., Behr, E.R., Mallmann, F.J.K., Graça, D.L., et al., 2006. Ion levels in the gastrointestinal tract content and plasma of four teleosts with different feeding habits. Fish Physiol. Biochem. 32, 105–112.

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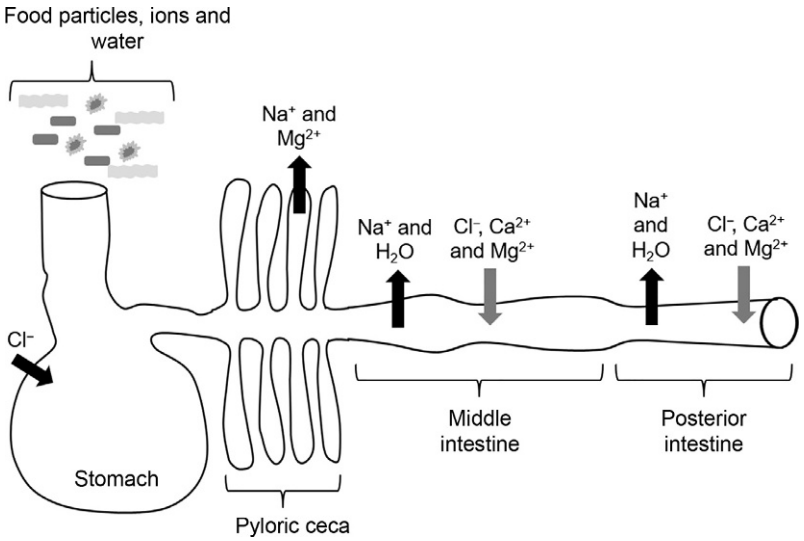

FIG. 12.4 Ion fluxes in the gastrointestinal tract of traíra, *Hoplias malabaricus*. *Black arrows* indicate absorption; *gray arrows* indicate secretion.

Mimura, 1997), independently of the feeding habit (Baldisserotto et al., 2004b). These ionic levels in the vesicular bile may also change according to water quality, as registered for silver catfish (*Rhamdia quelen*) acutely exposed to hypoxia and high levels of ammonia. However, these changes are related to changes in plasma levels, and not because the gallbladder is related to osmoregulation. The increase of plasma ion levels is likely to increase ion levels in the hepatic bile, given that its ion levels are similar to those in plasma. In this way, the increase of ion levels in the hepatic bile leads to increased ion levels in the vesicular bile (Becker et al., 2009).

Renal system

Freshwater Teleosts have a typical vertebrate renal nephron that includes glomeruli, proximal tubules, distal tubules, and collecting ducts. The glomerulus and distal tubule are responsible for water excretion (Beyenbach, 2004). In these fish, there are a large number of well-developed glomeruli that promote blood filtration, resulting in abundant glomerular filtration (4–5 mL kg^{-1} h^{-1}). The proximal tubule reabsorbs filtered nutrients and divalent ions (Ca^{2+}, Mg^{2+}, and SO_4^{2-}) and also a small amount of monovalent ions (Na^+, Cl^-, K^+, and HCO_3^-) and water (Fig. 12.5). About 90% of the Na^+ and Cl^- reabsorption occurs in the distal tubule through $Na^+/2Cl^-/K^+$ and Na^+/H^+ cotransporters (Cutler and Cramb, 2008; Nishimura and Fan, 2003), probably aided by an Na^+ gradient created by the Na^+/K^+ ATPase in the basolateral membrane (Bolner et al., 2014). However, water is not reabsorbed as the distal tubule is impermeable to water because the cell junctions present a large expression of claudin-8 (Bagherie-Lachidan et al., 2009). Only 45% of the filtered water is reabsorbed in the renal tubules. Even with a large production of urine, it is retained for about 20–30 min in the urinary bladder before excretion. There is an additional reabsorption of Na^+ and Cl^-, but not water, in the urinary bladder. The resulting urine is quite diluted, allowing the fish to eliminate the excess water gain by osmosis in the gills (Bolner and Baldisserotto, 2007; Curtis and Wood, 1991).

Hypoxia and osmoregulation

Long-term exposure of freshwater fish to hypoxia (approximately 1 week) results in increased gill area and consequent increased oxygen uptake. The increased gill area understandably favors both loss of ions and water intake (Fernandes et al., 1999). Hypoxia also stimulates gill chemoreceptors, which leads to increased blood pressure (Milsom et al., 1999).

Exposure to hypoxia, in most studied species, induced the release of catecholamines (Reid and Perry, 2003). This release can cause a constriction of the arteriovenous route, raising the blood perfusion pressure in the branchial vessels and increasing lamellar pressure and flow. All these processes contribute to the widening or distorting of the tight junctions between gills cells (Sundin, 1999). The increase of branchial ventilation causes a high efflux of ions via the paracellular route (Gonzalez and McDonald, 1992, 1994). Silver catfish exposed to 2.5 mg L^{-1} dissolved oxygen levels increased Na^+ and Cl^- loss in the first 24 h of hypoxia compared to normoxic fish (Rosso et al., 2006). Na^+ and K^+ loss was also registered for wolf fish, but not for the facultative air-breather jeju, *Hoplerythrinus unitaeniatus*, exposed for 1–3 h to severe hypoxia (0.75–1.5 mg L^{-1} dissolved oxygen levels). Jeju is able to sustain ionoregulation in hypoxia by increasing intestinal ion absorption (Wood et al., 2016). The Amazonian pacu *Metynnis hypsauchen* dies when maintained for 4 h in chambers with

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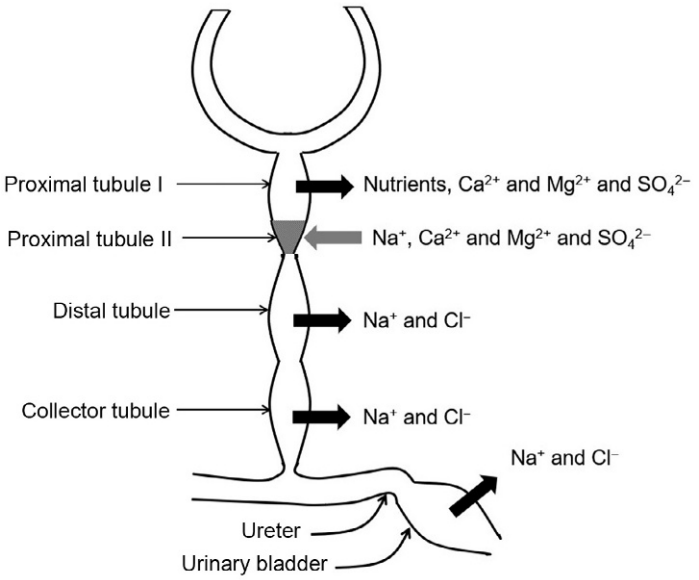

FIG. 12.5 Representative scheme of the renal system of freshwater Teleosts. *Black arrows* indicate reabsorption of nutrients, ions, and water; *gray arrow* indicates ion secretion. *(Adapted from Larsen, B.K., Perkins, E.J., 2001. Target organ toxicity in the kidney. In: Schlenk, D., Benson, W.H. (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts. Taylor & Francis, London, 91–150.)*

2–3 mg L^{-1} dissolved oxygen and presents increased net efflux of Na^+ and Cl^- at 3.5–4.0 mg L^{-1} DO levels compared to specimens maintained in normoxia (Baldisserotto et al., 2008b). Another strategy is observed in the oscar, *Astronotus ocellatus*. When exposed to severe hypoxia (0.3–0.7 mg L^{-1} dissolved oxygen [DO] levels) for 1 h, body Na^+ influx and efflux rates decreased, but net flux was not altered. The reduction of gill Na^+/K^+ ATPase activity and an increase on Na^+, Cl^-, and Ca^{2+} plasma levels was observed only after 3 h of hypoxia (Wood et al., 2007, 2009). The breathing rate in this species was not affected by hypoxia, but the total ventilation rate increased due to a rise in ventilatory stroke volume (Scott et al., 2008; Wood et al., 2009). Lower Na^+ and Cl^- net effluxes compared with specimens maintained in normoxia were also observed in the black piranha *Serrasalmus eigenmanni* exposed to 2.75–3.5 mg L^{-1} DO levels (Baldisserotto et al., 2008b) and tambaqui (Na^+ and K^+ net effluxes) exposed to 1.5–2.0 mg L^{-1} DO levels (Robertson et al., 2015). The oscar, *Astronotus ocellatus*, exposed to severe hypoxia has reduced transcellular gill permeability resulting from expansion of pavement cells and partial covering of neighboring ionocytes, which are recessed and reduced in size. Those remaining open were transformed from "shallow-basin" to "deep-hole" forms with smaller openings, deeper apical crypts, and smaller numbers of subapical microvesicles (Matey et al., 2011). After 20 h of exposure to these hypoxic levels, the activity of gill Na^+/K^+ ATPase was still low and plasma levels of Na^+, but not Cl^-, decreased. The authors hypothesized that hemoconcentration and a passage of water from the plasma to the extracellular medium would be the cause of an increase in plasma Na^+ (Richards et al., 2007). This hypothesis would also explain the increase in plasma ion levels registered by Becker et al. (2009) for silver catfish exposed to mild hypoxia (3.5 mg L^{-1} DO levels) for a similar period. Silver catfish kept for 48 h under hypoxia did not show any difference in Na^+ and Cl^- fluxes compared to fish maintained in normoxic waters, perhaps because of a reduction in the metabolism in those exposed to hypoxia. It is possible that this transient change in Na^+ and Cl^- fluxes caused a decrease in the plasmatic levels of these ions, which may have caused intracellular K^+ outflow and a consequent increased loss of K^+ 48 h after transfer (Rosso et al., 2006).

Severe hypoxia also reduces the glomerular filtration rate, urinary flow, and Na^+ reabsorption in the renal tubules of oscar (Wood et al., 2009). Contrary to that registered for black piranha, the Amazonian pacu presents an acidification of blood pH when in hypoxia, which could inhibit gill ion transporters (Baldisserotto et al., 2008b).

Two species from the Negro River, the cardinal tetra, *Paracheirodon axelrodi*, and the red nose tetra, *Hemigrammus rhodostomus*, exhibited negligible ionoregulatory responses to 2 h of hypoxia (1.0–1.6 mg L^{-1} DO levels), indicating that there may be a third pattern of osmoregulatory strategy during hypoxia or that the hypoxia level or time of exposure tested were not enough to induce a significant response in these species (Robertson et al., 2015).

Osmoregulation and pH

In general, Brazilian fish present 100% survival in waters with pHs between 4.0 and 9.0 (Baldisserotto, 2011), but the cardinal tetra, which lives in the black and acidic waters of the Amazon, thrives from pH 2.9 up to 8.8 (Oliveira et al., 2008). Survival at alkaline pH also seems to depend on prior adaptation to high pH values because in juvenile silver catfish

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previously adapted to pH 6.8, 50% of the fish died at pH 9.5 after 96 h (Zaions and Baldisserotto, 2000), whereas in those maintained at pH 7.5 before experimentation, no mortality was registered at the same pH (Townsend and Baldisserotto, 2001). However, in both cases, mortality was high at pH 9.75.

Fish living in acidic and diluted waters have difficulty maintaining ionic balance. The excess H^+ present in acidic waters competes with the Ca^{2+} and Na^+ of the water, inhibiting their capture by the fish. At the same time, H^+ loosens the paracellular protein junctions of the gill membrane so that the ion loss to the medium increases by diffusion. Silver catfish kept at pH 5.5 had a reduced gill respiratory surface area and an increased number of ionocytes in the gill filaments, but epithelium thickness was not affected (Costa et al., 2017). Tambaqui maintained at pH 4.0 for 40 days had reduced plasma Na^+ and K^+ concentrations when compared to those maintained at pH 6.0 and 8.0 (Aride et al., 2007). Reduction of the environmental pH would lead to a decrease in blood pH as a result of the inhibition of Na^+/H^+, Cl^-/HCO_3^-, and V-ATPase in the gills (Wood, 2001). Reduction of blood pH, in turn, triggers an increase in urinary excretion of H^+ and NH_4^+, reducing urine pH to compensate for this problem (Bolner and Baldisserotto, 2007). Acute exposure to acidic or alkaline waters reduced plasma Na^+ and Cl^- levels in pirapitinga, *Piaractus brachypomus* (Garcia et al., 2014), plasma Cl^- levels in silver catfish (Bolner and Baldisserotto, 2007), and also increased plasma K^+ levels in both species (Costa et al., 2015; Garcia et al., 2014). Silver catfish exposed for 3–4 days to acidic pH showed lower plasma and body Na^+, but no alteration in Cl^- levels (Costa et al., 2015; Zaions and Baldisserotto, 2000). The difference in the response of plasma Na^+ and Cl^- levels to acidic pH in silver catfish may be linked to their distinct ionoregulatory mechanisms across the gill epithelium (Costa et al., 2015).

In order to live in the acidic waters of the Negro River, the fish present two strategies of osmoregulation. Characins have transporters with a high affinity for Na^+ that are not affected by acidic pH, and with high rates of uptake and loss of this ion. On the other hand, Cichlids developed carriers with low affinity for Na^+, providing low absorption rates. Cichlids are also sensitive to acidic pH, but fish also exhibit a reduced loss of ions by diffusion, which does not increase at acidic pH (Gonzalez et al., 2002; Gonzalez and Wilson, 2001). There is also reduction in H^+-ATPase activity, but not in Na^+/K^+-ATPase activity, in the gills of angelfish (*Pterophyllum scalare*) (Duarte et al., 2013). The freshwater stingray, *Potamotrygon* sp., also fits the latter category (Wood et al., 2002). The neon tetra, *Paracheirodon innesi*, even when exposed to water at pH 3.25, does not present inhibition of Na^+ and Cl^- influx, and does not seem to use the Na^+/H^+ antiporter and H^+-ATPase (V-ATPase) in the gills, although the antiporter Cl^-/HCO_3^- was detected. Most of the ion uptake mechanisms of this species have not yet been determined (Preest et al., 2005).

Tambaqui exposed to pH 8.0 experienced increased net acidic equivalent excretion and/or basic equivalent uptake, which occurred mainly at the gills, provoking an increase of blood pH (Wood et al., 2018). The increase of blood pH was also registered for silver catfish exposed to alkaline pHs (Bolner and Baldisserotto, 2007); exposure to pH 8.0 also increased net Cl^- loss, but net Na^+ balance was little affected (Wood et al., 2018).

Water hardness and osmoregulation

The content of Ca^{2+} and Mg^{2+} salts, closely linked to carbonate (CO_3^{2-}), bicarbonate (HCO_3^-), and SO_4^{-2} ions, chlorides, and other anions of mineral acidity determine the water hardness (Baldisserotto, 2011). The sources of internal Ca^{2+} reserves (bone) in fish are poorly accessible, so the regulation of plasma Ca^{2+} depends basically on the ingestion of this ion through feed or its uptake via gills (Baldisserotto et al., 2004a,b).

In soft water conditions, there is an increase in the number of ionocytes and in some species also of its apical surface to increase the uptake of Ca^{2+}. In the jeju, the transfer from water with 1.7 mg $CaCO_3$ L^{-1} to deionized water increased the number of ionocytes, but these cells are "buried" between pavement cells and the apical surface has a spongy structure, developing an apical ridge. These adaptations would promote a microenvironment within the crypt, preventing ion loss and facilitating their uptake in an extremely diluted medium (Moron et al., 2003). When maintained in soft water (about 10–30 mg $CaCO_3$ L^{-1}), wolf fish rarely exhibit ionocytes in the lamellae, but jeju, which has accessory aerial respiration, has a good number of these cells in the lamellae. The number of ionocytes in the filaments and lamellae increases in both species when exposed to distilled water, but the surface of these cells in contact with the environment and the number of mucous cells do not change in any of the species (Moron et al., 2003, 2009). The number of ionocytes also increased in the gills of the *Hypostomus tietensis* exposed to distilled water (Fernandes and Perna-Martins, 2002). The increase in the number of ionocytes in the lamellae and the increase in the thickness of the lamellar epithelium that also occurs in this situation hindered the gas exchange, so that the partial oxygen pressure in the blood decreased even in normoxia. In such conditions, fish present increased branchial ventilation to avoid the drop of the blood partial pressure of oxygen. In a situation of hypoxia in distilled water, there is an increase in the release of catecholamines, resulting in increased blood flow to the gills (Sakuragui et al., 2003).

Specimens of the ray *Paratrygon aiereba* collected in the Negro River present lower plasma Na^+, Cl^-, urea levels, and osmolality as well as higher gill and renal Na^+/K^+ ATPase activity than those collected in the Amazon River, with consequent increased branchial uptake of ions and decreased kidney loss in ion-poor water (Duncan et al., 2009). Pirarucu and

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tamoatá, *Hoplosternum littorale*, transported from the black water of the Negro River to the white water of the Solimões River (which has about 10 times more Ca^{2+}) and vice versa, presented only slight changes of net ion fluxes, but both species showed great ion loss when placed in acidic black waters (pH 3.5) (Baldisserotto et al., 2008a).

The increase of water hardness at least up to 600 mg $CaCO_3$ L^{-1} (higher values were not tested yet) for 96 h did not alter the survival of silver catfish juveniles at neutral, slightly acidic, and alkaline pHs, but at extreme pHs the addition of Ca^{2+} increases survival (Townsend and Baldisserotto, 2001).

Dissolved organic matter and osmoregulation

Several water bodies such as the Rio Negro (AM, Brazil) contain low concentrations of ions (Table 12.1), acidic pH, and a large amount of dissolved organic matter (Aucour et al., 2003; Mortatti and Probst, 2003). This organic matter consists basically of humic substances (HS), which are organic compounds generated from the deterioration of plant and animal remains that have functional acid groups, such as the carboxylic and phenolic-hydroxyl groups (Thurman, 1985). It has been reported that HS interacts with biological membranes directly affecting living organisms (Campbell et al., 1997; Steinberg, 2003), probably as a result of hydrophobic interactions between membrane lipids and the hydrophobic domains of HS.

Teleosts and the ray *Potamotrygon* sp. kept in natural black water had lower ion efflux rates after exposure to low pH (3.5–4.0) compared to fish kept in ion-poor water and without HS, suggesting that HS reduces gill permeability to diffusional ion loss (Gonzalez et al., 1998, 2002; Wood et al., 2002, 2003). In cardinal tetras exposed to pH 3.75, the HS (35 mg carbon L^{-1}) reduced Na^+ loss by diffusion and increased Ca^{2+} uptake (Matsuo and Val, 2007). However, in silver catfish, the gill filament epithelium thickness and lamellar wideness increased with increasing levels of commercial humic acid (10–50 mg L^{-1}) (Costa et al., 2017). The presence of the commercial humic acid did not alleviate the ionoregulatory and/or acid-base challenge prompted by low pH; instead, it impaired osmoregulation (Costa et al., 2017).

Conclusions

Given that there are no more than a few studies evaluating the mechanisms of osmoregulation in Neotropical fish, except for silver catfish and some Amazonian species, it seems opportune to foster studies on the subject. Knowledge on osmoregulation of Neotropical species in alkaline waters and hard waters (at any pH) is nothing but scarce. In addition, the determination of ionic levels in the digestive tract of fish collected directly from their natural environment can also increase knowledge about osmoregulation, especially in ion-poor waters, where it is assumed that a good part of the ion input must be by feeding.

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Chapter 13

Reproduction and embryogenesis

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General overview

Fish exhibit great diversity of species with the most varied life cycle patterns among vertebrates. In South America, teleosts have achieved success in different environments due to a wide spectrum of reproductive strategies and extreme tactics. Most freshwater fish species are iteroparous (i.e., they spawn more than once in their lives), gonochoristic, (i.e., sexes are separated in different specimens), and exhibit external fertilization without parental care. Despite species diversity, freshwater ichthyofauna are still underexplored because several aspects of their biological cycle are still unknown or have been little studied. This lack of biological knowledge makes it difficult to develop breeding technologies for the main commercially important species and to understand the evolutionary history of the neotropical ichthyofauna as well as the phylogenetic relationships among its groups.

Reproduction is one of the most important aspects of fish biology, and its success depends on the spawning and recruitment of fingerlings, essential events in the sustainability of fish populations in wild environments. In Brazil, studies on fish reproduction began with the works of Ihering (von Ihering, 1928, 1933; von Ihering and Wright, 1935); however, these studies intensified after 1960 when hydroelectric dams were built in the main hydrographic basins in southeastern Brazil, especially the Três Marias dam in the Upper São Francisco River and the Furnas dam in the Grande River, Upper Paraná River basin.

Interactions between endogenous and environmental factors are critical for triggering spawning and for the reproductive success of neotropical fish. Environmental stimuli, especially rain, temperature, and photoperiod, act on the hypothalamus, inducing the production of the gonadotrophin-releasing factors while stimulating the release of gonadotrophic hormones and the production of sex steroids, which are responsible for gamete maturation (Nagahama et al., 1995; Nagahama and Yamashita, 2008). Failure in reproduction for consecutive years, caused mainly by habitat changes, can lead to the depletion of natural fish stocks and species extinction.

In tropical regions, most fish reproduction occurs in the rainy season when temperature, photoperiod, and water flow increase, acting as a trigger for migration and spawning. Several species of commercial importance go upriver to reproduce in their headwaters (a phenomenon known as *piracema*), and then their eggs are carried to places favorable to the survival and development of the eggs and larvae (Lowe McConnel, 1987).

Hydroelectric dams interrupt the migratory routes of fish and alter reproductive activity both upstream and downstream, especially of migratory species. Changes in the hydrological regime are observed downstream of dams because of a

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decrease in flood peaks, resulting in unstable thermal and hydrodynamic conditions that negatively affect fish reproduction (Sato et al., 2005). In the São Francisco River, the females of curimatã *Prochilodus argenteus* do not reach final oocyte maturation, and their plasma sex steroid levels and fecundity decrease in the stretch immediately after the Três Marias dam (Arantes et al., 2010). Females with mature ovaries of lambaris *Astyanax bimaculatus* and *A. fasciatus*, matrinxã *Brycon orthotaenia*, and piaus *Leporinus reinhardi* and *Schizodon knerii* were also not captured in this river section (Brandão et al., 2017; Normando et al., 2014; Nunes et al., 2015; Weber et al., 2013a, b). Similar results were obtained with curimatã *Prochilodus lineatus* downstream from the Porto Colombia dam in the Grande River, upper Paraná River basin (Perini et al., 2013). Upstream of the dams, the transformation of a river section (lotic environment) into a reservoir (lentic environment) modifies the ichthyofauna profile, favoring the establishment of species that do not depend on floods as triggers for spawning. In the Três Marias reservoir, located upstream of the hydroelectric power dam, the collapse of the reproductive process of two species of migratory fish, curimatã *Prochilodus costatus* and *P. argenteus,* has been reported (Arantes et al., 2011a).

When kept in captivity, migratory species of high economic value do not reproduce naturally because their gonads develop until advanced maturation, but hormonal induction is needed for females to achieve final oocyte maturation and spawning. Induced spawning by hypophysation has been successfully performed in several commercially important species of the São Francisco and Paraná River basins using a common carp crude pituitary extract (Arantes et al., 2013; Leonardo et al., 2004; Santos et al., 2013a, b; Sato et al., 2000, 2003b). Although Brazil is a pioneer in the induced reproduction of freshwater fish, hormonal induction protocols have not yet been established for many commercially important native species. In addition, detailed studies of reproductive biology and early development are still needed to improve breeding techniques and to establish conservation strategies for native ichthyofauna.

Structural organization of the gonads

In most Neotropical species, including the three major groups of teleosts in South America, that is, Characiformes, Siluriformes and Perciformes, the ovaries and testes are paired organs located in the coelomic cavity laterally to the gas bladder and dorsally to the digestive tract (Martins et al., 2012; Melo et al., 2011). In the caudal region, the right and left gonads unite to form a common duct that opens into the urogenital papilla, caudally to the anal opening.

Microscopically, testicular organization is common in most teleost fish (Grier, 1981). Testes are enclosed by a tunica albuginea that emits septa into the organ, delimiting the tubular and interstitial compartments. The tubular compartment is constituted by germ cells associated with Sertoli cells forming cysts, where isogenic cells develop synchronously. At the base of the seminiferous tubules is the basal membrane synthesized by Sertoli cells, and myoid peritubular cells, which support the development of germ cells. In the interstitial compartment are connective cells and fibers, blood vessels, immune cells, and Leydig cells producing sex steroid hormones.

According to tubular compartment organization, the testes have been classified as anastomosed tubular with branched seminiferous tubules interconnect, and lobular with germ cell cysts organized into lobes (Parenti and Grier, 2004). Regarding spermatogonia distribution, the testes can be classified into two types: (a) unrestricted spermatogonial, which has spermatogonia throughout the seminiferous tubule; and (b) restricted spermatogonial, in which the spermatogonia are concentrated in the distal portion of the tubule (Grier, 1981). The anostomosed tubular and unrestricted spermatogonial patterns have been frequently detected in neotropical fish, as reported for several species of Characiformes and Siluriformes (Arantes et al., 2011a, b; Carvalho et al., 2009; Lopes et al., 2004; Magalhães et al., 2004; Martins et al., 2010; Martins et al., 2012; Melo et al., 2011, 2017; Santos et al., 2001). Lobular organization with spermatogonia concentrated in the inferior portion of the testes was detected in the Nile tilapia *Oreochromis niloticus* (Melo et al., 2016).

In Siluriformes, the testes present lobes with or without digitiform projections or fringes, especially in the Pimelodidae family. The testicular fringes develop during gonadal maturation, becoming very evident during the spawning season, as observed in the mandis *Pimelodus maculatus* and *Iheringichthys labrosus* (Guimarães-Cruz and Santos, 2004; Santos et al., 2001), the pirá *Conorhynchus conirostrus* (Lopes et al., 2004), and the pseudopimelod pacamã *Lophiosilurus alexandri* (Barros et al., 2007). However, some species do not present testicular fringes such as the armored catfish *Hoplosternum littorale* (Loir et al., 1989), *Rhinelepis aspera* (Agostinho et al., 1987), and *Loricaria lentiginosa* (Guimarães-Cruz et al., 2005) from the families Callichthyidae and Loricariidae. Seminal vesicles were found in the driftwood catfish from the Auchenipteridae family *Trachelyopterus galeatus*, a species with internal fertilization (Loir et al., 1989; Meisner et al., 2000; Melo et al., 2011; Santos et al., 2013a).

Microscopically, ovaries are coated by the tunica albuginea, which emits septa into the organ, forming ovigerous lamellae where oocytes develop. Three oocyte development patterns have been found in teleosts (de Vlaming, 1983): (1) synchronous, when all oocytes develop simultaneously, which is associated with semelparous species that spawn only

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once in their lives and die, such as the Pacific salmon (*Oncorhynchus* sp), lampreys, and eels (*Anguilla* sp); (2) group synchronous, when oocytes develop in two distinct groups, one of them being a reserve stock group for the next reproductive cycle. This pattern is characteristic of species that spawn in a short spawning season, such as the dourados *Salminus brasiliensis* and *S. franciscanus*, the catfish *Pseudoplatystoma corruscans*, the curimatãs *Prochilodus costatus* and *P. argenteus*, and the pacu *Piaractus mesopotamicus*, among others; and (3) asynchronous, characterized by the presence of more than two lots of oocytes at various phases of development. This pattern is associated with species that spawn several times in a prolonged spawning season, including the piranhas *Pygocentrus piraya* and *Serrasalmus brandtii* and the lambaris *A. bimaculatus* and *A. fasciatus*, among others.

The functional unit of the ovary is the follicular complex (Grier, 2000), which consists of two compartments separated by a basal membrane. The germinal compartment contains the oocyte surrounded by follicular cells, separated by an extracellular matrix, the zona radiata. Prolongations of the oocyte and follicular cells interdigitate and form the pore canals in the zona radiata. Unlike mammalian granulosa cells, the follicular cells do not develop a corpus luteum in the fish ovary. Externally to the basal membrane is the theca constituted of connective tissue, which originates from the ovarian stroma and has smooth muscle cells that assist in oocyte elimination during ovulation. The development of ovarian follicles follows a general pattern in teleosts, but its morphology varies according to the reproductive strategy of the species (Martins et al., 2010; Melo et al., 2011, 2017). During folliculogenesis, morphological changes occur in the oocyte and follicular envelope: zona radiata, follicular cells, and theca. Some species have structures associated with the zona radiata, that is, filaments, globules, and villus, which are related to egg adhesiveness (Rizzo et al., 2002). In Siluriformes, a gelatinous coating (jelly coat) is observed covering the zona radiata in both the adhesive and nonadhesive eggs of the Pimelodidae and Loricaridae families (Rizzo et al., 2002; Sato et al., 2003a, b).

First sexual maturation

The size at first sexual maturation is the smaller size at which fish reach the ability for reproduction. It is an important parameter of fish reproductive biology because it is considered in fishing regulations, especially for commercially important species. However, this size has been investigated in few neotropical species due to the difficulty in capturing and identifying immature fish in the wild.

Size at first sexual maturation is commonly determined by the L_{50} method, which establishes the length at which 50% of the sampled specimens are adult and 50% are immature or juvenile. This value represents the minimum size needed to avoid capturing young specimens and helps establish the minimum size of net meshes to be used in commercial fishing (Vazzoler, 1996). The L_{100} indicates the size in which all specimens are adult, that is, apt to reproduction, and it is important for induced reproduction procedures in fish farming.

The distinction between immature fish and resting is difficult when using only macroscopic criteria. However, histological analyses show that the ovaries of immature fish do not have a defined lamellar organization, and the oogonia and perinucleolar oocytes are distributed in a compact arrangement (Rizzo et al., 1996). Ovaries of juvenile fish have lamellar organization and a compact arrangement of oocytes. Testes of immature fish have abundant interstitial stroma with spermatogonia randomly distributed and do not present evident tubular organization (Cruz et al., 1996; Soares et al., 1996). In the testes of juvenile fish, seminiferous tubules are organized with numerous spermatogonia but without an evident lumen, and the interstitial stroma is scarce. The histological features of the ovaries and testes of immature and juvenile fish are shown in Fig. 13.1A–D.

In general, Characiformes males reach their first sexual maturation with a smaller size than females, as found in *S. brandtii* (Honorato-Sampaio et al., 2009), *Leporinus piau*, *L. reinhardti*, and *S. knerii* (Rizzo et al., 1996; Soares et al., 1996). These variations between sexes reflect sexual dimorphism while the intraspecific variations in the same sex can be determined by the availability of food in different habitats, thus causing different growth rates (Nikolsky, 1963). The values of L_{50} and L_{100} for some fish species from the São Francisco River basin are shown in Table 13.1. In these species, the size at the first sexual maturation was about 60% of the size of the largest specimen captured in the wild (Cruz et al., 1996; Honorato-Sampaio et al., 2009; Rizzo et al., 1996; Soares et al., 1996). In order to avoid capturing immature and young fish, the size at first sexual maturation can be estimated with reference to the smallest specimen caught at the advanced maturation stage (Bazzoli, 2003), thus avoiding the capture of immature and young fish in the wild.

Gonadal maturity stages

Macroscopic and microscopic analyses of gonadal maturation are important to understand the reproductive behavior of a fish species in its habitat. Different gonadal maturity scales have been proposed when studying the reproductive cycle of

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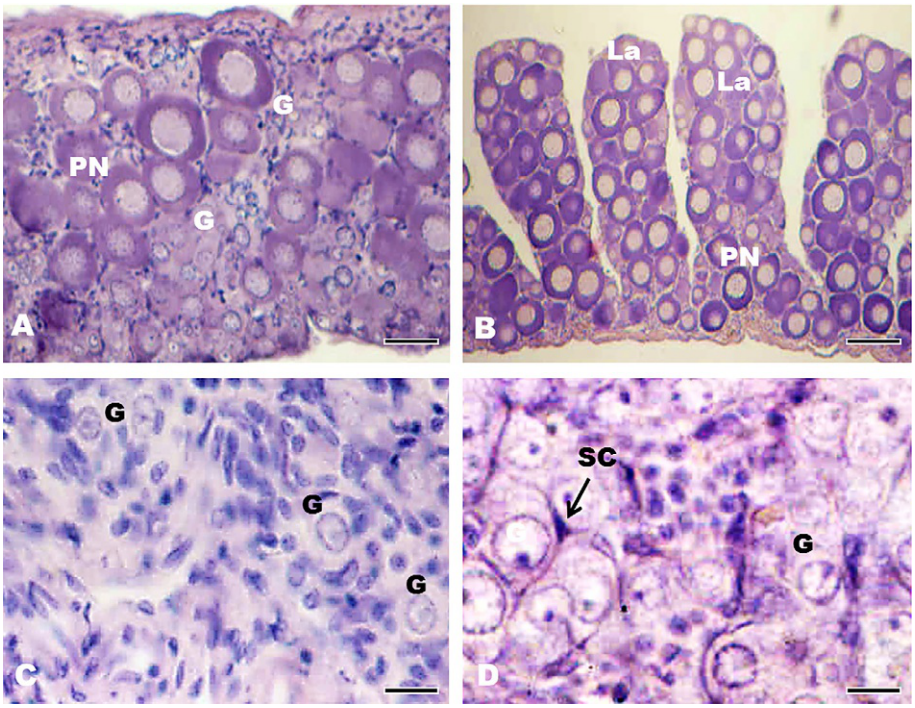

FIG. 13.1 Histological sections of gonads during gonadal differentiation. (A) Immature ovary with oogonia nests (G), perinucleolar oocytes (PN), and without lamellar organization. (B) Juvenile ovary with lamellar organization and compact arrangement of oocytes. (C) Immature testis with some spermatogonia (G) and undifferentiated cells, without tubular organization. (D) Juvenile testis with spermatogonia (G), Sertoli cells (SC), and seminiferous tubules in formation, and no visualized tubular lumens. Bars: (A) 100 μm, (B) 132 μm, (C, D) 9 μm.

TABLE 13.1 Size (cm) at first sexual maturity (L_{50}) and size when all specimens are adult (L_{100}) in fish species from the São Francisco River basin, Brazil.

Species/Authority	Females			Males			References
	SL	L_{50}	L_{100}	SL	L_{50}	L_{100}	
Characiformes							
Leporinus piau (Fowler, 1941)	13	10.2	12.0	12	9.6	10.8	Soares et al. (1996)
Leporinus reinhardti (Lütken, 1875)	18	14.0	16.9	18	12.9	15.1	Rizzo et al. (1996)
Pygocentrus piraya (Cuvier, 1819)	38	17.0	28.0	34	10.8	18.0	Cruz et al. (1996)
Schizodon knerii (Steindachner, 1875)	20	14.5	17.5	18	12.3	14.7	Soares et al. (1996)
Serrasalmus brandtii (Lütken, 1875)	18	14.5	n. d.	16	13.1	n. d.	Honorato-Sampaio et al. (2009)
Perciformes							
Pachyurus squamipennis (Agassiz, 1831)	35	21.4	26.5	n. d.	n. d.	n. d.	Andrade et al. (1996)

SL, mean standard length (snout to the end of the caudal fin) of the species; L_{50}, SL at first sexual maturity; L_{100}, SL size when all fish are adults; *n.d.*, nondetermined.

freshwater fish, depending on the species, sample size, and methodology utilized. These variations do not reflect morphological or functional differences in the gametogenesis, which is similar among teleosts (Martins et al., 2010; Melo et al., 2011, 2017; Selman and Wallace, 1989). In general, the most frequently used gonadal maturity stages are resting, early ripening, late ripening, ripe, partially spawned for females and partially spent for males, and fully spawned or spent. The main features of these stages are found in Table 13.2, and Figs. 13.2A–F and 13.3A–F.

In the piaba *Bryconops affinis*, the resting stage was not detected in males and females captured bimonthly at the Três Marias dam during two consecutive reproductive cycles, suggesting continuous gametogenesis in this species (Andrade et al., 2001). Resting males were also not found in other Characidae species such as the dogfish *Acestrorhynchus lacustris*

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TABLE 13.2 Macro- and microscopical features of gonads in different reproductive cycle stages.

Stage	Ovaries	Testes
Stage 1 Resting (Figs. 13.2A and 13.3A)	Thin and translucid ovaries, ovigerous lamellae containing oogonia nests and perinucleolar follicles	Thin and translucid testes, seminiferous tubules with spermatogonia and closed lumen
Stage 2A Early ripening (Figs. 13.2B and 13.3B)	Ovaries with increased volume and color characteristic of the species, ovigerous lamelas have perinucleolar and previtellogenic follicles with cortical vesicles	Increased volume and whitish testes, seminiferous tubules have numerous cysts of germ cells and small amount of spermatozoa
Stage 2B Late ripening (Figs. 13.2C and 13.3C)	Voluminous ovaries with oocytes visible to the naked eye, ovigerous lamellae with perinucleolar, previtellogenic and vitellogenic follicles	Maximum volume with milky white colour, seminiferous tubules with wide lumen full of spermatozoa
Stage 3 Ripe (Figs. 13.2D and 13.3D)	Similar to the previous stage; however, some vitellogenic oocytes show migration of the germinal vesicle toward the micropyle, evidence of the final oocyte maturation. Mature females can release eggs spontaneously or under gentle pressure at the coelomic wall	Similar to the previous stage; however, spermatozoa are found in the tubular lumen and spermatic duct. Mature males can release semen under gentle pressure in the coelomic wall
Stage 4A Partially spawned/spent (Figs. 13.2E and 13.3E)	Ovaries are flaccid with hemorrhagic areas and oocytes visible to the naked eye. Presence of perinucleolar, previtellogenic, vitellogenic, and postovulatory follicles	Testes are flaccid with milky white areas, seminiferous tubules have germ cells cysts and partially emptied lumen, with fewer spermatozoa
Stage 4B Fully spawned/spent (Figs. 13.2F and 13.3F)	Reduced, flaccid, and hemorrhagic ovaries, with atretic follicles (opaque) visible to the naked eye. Presence of perinucleolar, postovulatory, and atretic follicles	Testes are completely flaccid and hemorrhagic, seminiferous tubules are fully emptied, and may have residual spermatozoa

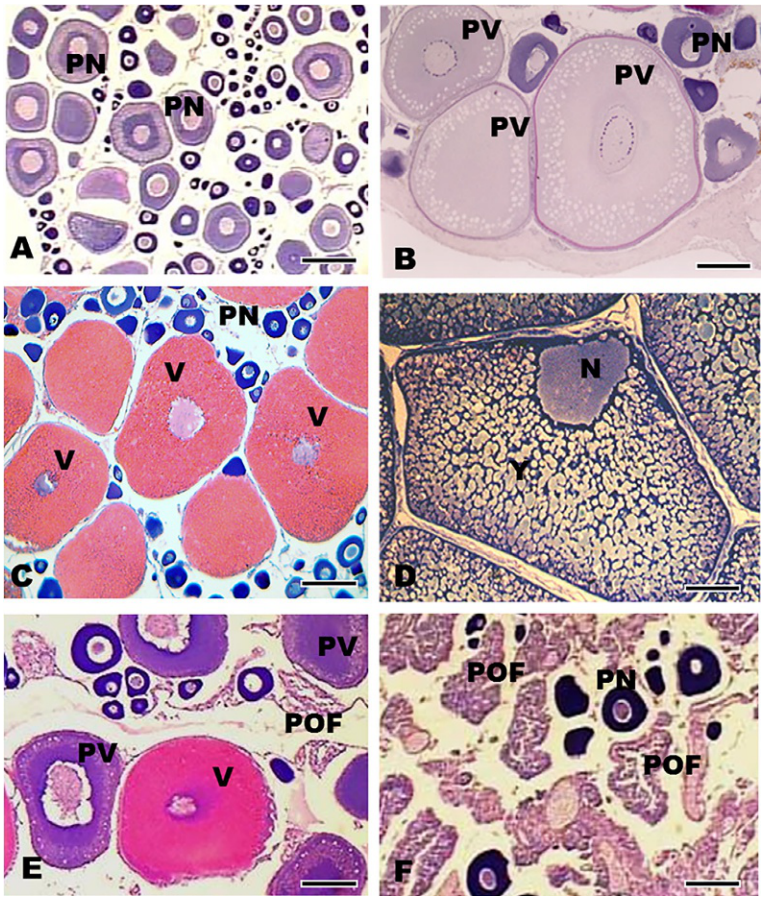

FIG. 13.2 Ovarian maturity stages: (A) resting, (B) early ripening, (C) late ripening, (D) ripe, (E) partially spawned, and (F) totally spawned. (PN) perinucleolar, (PV) previtellogenic follicle with cortical alveoli, (V) vitellogenic follicle, (N) nucleus displaced toward to micropyle, feature of the final oocyte maturation, (Y) yolk, (FPO) postovulatory follicle, (FA) atretic follicle. Bars: (A, B) 250 µm, (C) 160 µm, (D) 125 µm, (E) 278 µm, (F) 313 µm.

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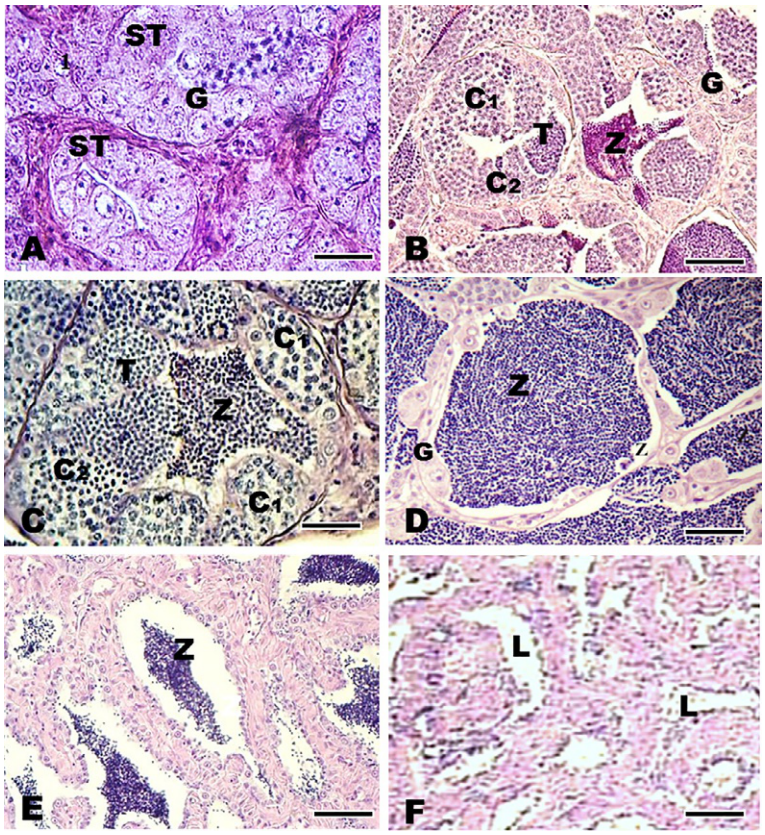

FIG. 13.3 Testicular maturity stages: (A) resting, (B) early ripening, (C) late ripening, (D) ripe, (E) partially spent, and (F) totally spent. (ST) seminiferous tubule, (G) spermatogonia, (C1) primary spermatocytes, (C2) secondary spermatocytes, (T) spermatids, (Z) spermatozoa, (L) tubular lumen. Bars: (A) 83 μm, (B) 154 μm, (C) 120 μm, (D) 167 μm, (E) 58 μm, (F) 50 μm.

(Bazzoli and Godinho, 1991); the forage fish from the Três Marias dam, that is, *Anchoviella vaillanti*, *Hemigrammus marginatus*, *Moenkhausia costae*, and *Roeboides xenodon* (Bazzoli et al., 1997); or the pirambeba *S. brandtii* (Honorato-Sampaio et al., 2009). Different from the temperate climate regions, a continuous or prolonged pattern of reproductive activity is common in tropical ecosystems, where the seasons are not as well defined (Lowe McConnel, 1987). During ripening, the ovaries and testes progressively increase in volume, and the germ cells become mature. The ripe stage, which is the time of ovulation or spermiation, is a quick process that is difficult to detect in fish caught in the wild, especially females. Therefore, the ripe stage has often been associated with the late ripening stage in several studies (Bazzoli et al., 1991; Bazzoli and Godinho, 1991; Honorato-Sampaio et al., 2009; Rizzo et al., 1996, and others). When found in the wild, ripe females spawn on light pressure of the coelomic cavity, as observed in commercially important species from the São Francisco River (Arantes et al., 2010; Sato et al., 2005). Nuclear migration toward the micropyle is histological evidence of the final oocyte maturation, which is the main event of the ripe stage. This event has been observed when females are submitted to hormonal induction to spawn (Rizzo et al., 1997). The spawned stage is characterized by the presence of postovulatory follicles, which are remnant structures of ovulated follicles, and by the atretic follicles, which are responsible for the reabsorption of nonovulated follicles.

Biological indices

Biometric parameters such as total length (TL) and body weight (BW) as well as the gonadosomatic index (GSI), the hepatosomatic index (HSI), the stomach repletion index (SRI), and condition factor (K) are important to evaluate the way the species get resources from the habitat. They also indicate the physiological condition of the specimens based on fat accumulation, gonadal development, general well-being, and adaptation to the environment (Nikolsky, 1963). In the field, standard length (SL), which measures the length of the fish until caudal fin insertion, is used when it is not intact.

In fish, GSI is a good indicator of reproductive activity, so the spawning season is determined by an association of the GSI and the frequency distribution of the gonadal maturity stages. The HSI reflects liver participation in vitellogenesis. The gonadal weight (GW) and the liver weight (LW) are obtained to calculate the GSI (100 GW/BW) and HSI (100 LW/BW). The GSI follows gonadal maturation, reaching higher values at the ripe stage, and then decreasing after spawning and

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spermiation, especially in females (Fig. 13.4A). At the late ripening stage, GSI varies according to the reproductive strategies of the species, and in general, total spawning fish have higher GSIs than those with multiple spawnings. In addition, GSI is also related to egg size, so that *P. corruscans* has a lower GSI compared to other total spawners such as curimatã *P. argenteus*, mainly due to the smaller size of the egg. The relationship between GSI and the reproductive strategies of some Characiformes, Siluriformes, and Perciformes is shown in Table 13.3.

During gonadal maturation, vitellogenin (Vtg) and zona radiata proteins (Zrp) are synthesized by hepatocytes, then transported through the bloodstream to the ovaries by receptor-mediated pinocytosis (Arukwe and Goksøyr, 2003). Therefore, HSI increases at early ripening, then decreases at late ripening as detected in the piau *Schizodon nasutus* (Fig. 13.4B) and other species (Bazzoli et al., 1998; Ribeiro et al., 2006; Santos et al., 2004). In males, HSI does not follow the same tendency of females, and presents less expressive variations during the reproductive cycle. However, the cyclical variations of HSI are not always found in most fish species, probably due to other functions of the liver such as detoxification of substances in impacted environments.

The condition factor (K) expresses the ratio between fish body weight and length, reflecting the interactions between biotic and abiotic variables in the physiological condition of the fish (Le Cren, 1951). The Fulton condition factor assumes an isometric increase in weight as a function of fish length and is calculated using the following equation: $K = 100BW/TL^3$. In some species, K values tend to be higher in the resting and early ripening stages and lower during late ripening and spawning season (Fig. 13.4C). The allometric condition factor is expressed by $K = 100BW/TL^b$, where b is the angular coefficient of the ratio between body weight and length, which ranges from 2.5 to 4.0 among species (Rossi-Wongtschowski, 1977). To eliminate the gonadal maturation interference, K can be calculated by deducting the gonadal weight (GW) or viscera weight (VW) from the body weight: $K = BW - GW/TL^b$ and $K = BW - VW/TL^b$, respectively. The use of the allometric condition factor has been recommended, especially for species that do not show isometric growth (Vazzoler, 1996).

Variations in the coelomic fat content may be related to gonadal maturation, the hydrological cycle of the river or reservoir, and food availability. The coelomic fat index (CFI) expresses the ratio of coelomic fat weight (CFW) and body weight (CFI = 100 CFW/BW). In general, it is lower when fish consume fat reserves to reproduce, that is, stages 3 (ripe) and 4A (partially spawned/spent) (Fig. 13.4D). After the breeding season, higher food availability leads to an increase in

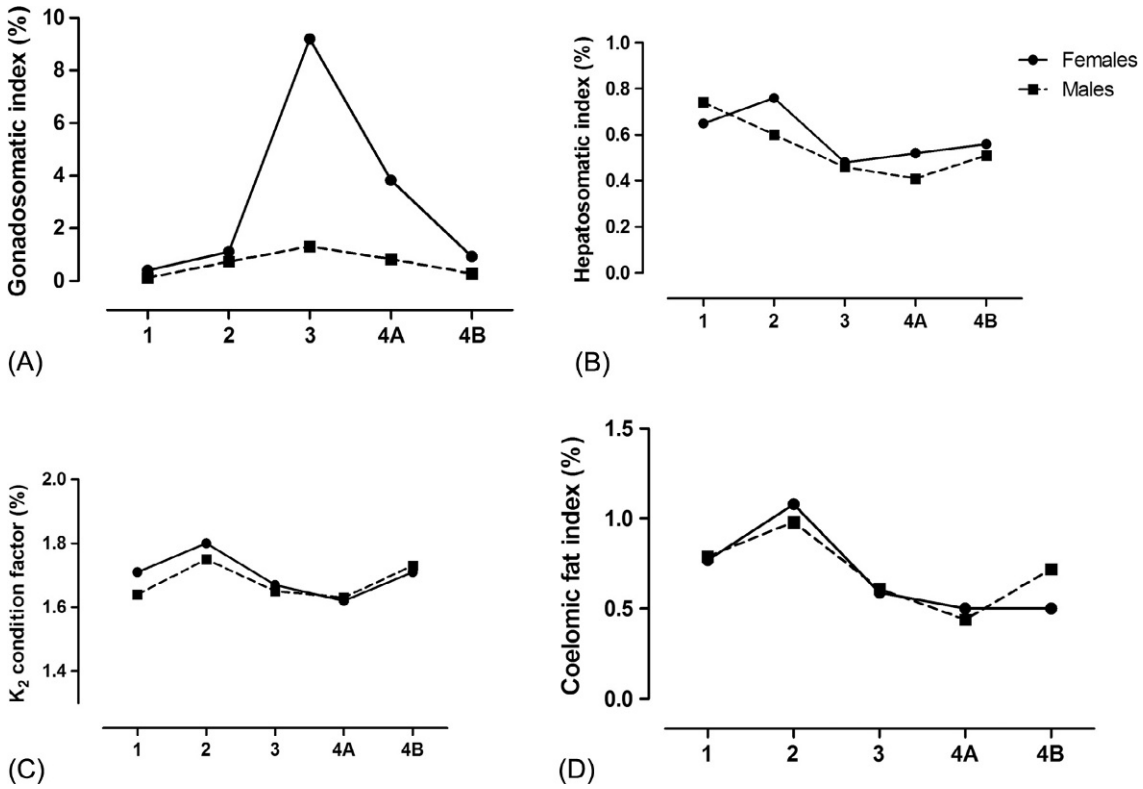

FIG. 13.4 Biological indices by gonadal maturity stage in piau *Schizodon nasutus* from the Estreito Reservoir, Grande River, southeastern Brazil. (A) Gonadosomatic index, (B) Hepatossomatic index, (C) Coelomic fat index, and (D) Fulton condition factor (K_2), excluding gonad weight.

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TABLE 13.3 Breeding season, spawning type and gonadosomatic index (GSI, mean ± DP) of ripe females in fish from the Grande and São Francisco Rivers basins, Brazil.

Species/Authority	River basin	Breeding season	Spawning	GSI	References
Characiformes					
Apareiodon affinis (Steindachner, 1879)	Grande	November to May	Multiple	14.6 ± 2.4	Ratton et al. (2003)
Acestrorhynchus britskii (Menezes, 1969)	São Francisco	Whole year	Multiple	5.5 ± 3.1	This chapter
Acestrorhynchus lacustris (Lütken, 1875)	São Francisco	Whole year	Multiple	5.4 ± 2.5	Bazzoli and Godinho (1991)
Astyanax bimaculatus (Linnaeus, 1758)	Grande	Whole year	Multiple	18.2 ± 2.0	Bazzoli et al. (1998)
Astyanax fasciatus (Cuvier, 1819)	Grande	Whole year	Multiple	15.0 ± 1.4	Carvalho et al. (2009), Prado et al. (2011)
Astyanax rivularis (Lütken, 1875)	São Francisco	Whole year	Multiple	8.5 ± 0.5	Weber et al. (2017)
Bryconops affinis (Günther, 1864)	São Francisco	Whole year	Multiple	7.8 ± 1.8	This chapter
Brycon orthotaenia (Günther, 1864)	São Francisco	October to February	Total	12.3 ± 2.1	Gonçalves et al. (2006)
Curimatella lepidura (Eigenmann and Eigenmann, 1889)	São Francisco	October to March	Total	5.7 ± 4.1	Alvarenga et al. (2006)
Galeocharax knerii (Steindachner, 1879)	Grande	Whole year	Multiple	8.2 ± 7.8	Magalhães et al. (2004)
Hemigrammus marginatus (Ellis, 1911)	São Francisco	Whole year	Multiple	5.3 ± 4.0	Bazzoli et al. (1997)
Leporinus obtusidens (Valenciennes, 1837)	São Francisco	November to February	Total	18.7 ± 1.6	Arantes et al. (2017), Sato et al. (2005)
Leporinus piau (Fowler, 1941)	São Francisco	December to April	Total	15.8 ± 1.7	Tavares and Godinho (1994), Arantes et al. (2017)
Leporinus reinhardti (Lütken, 1875)	São Francisco	November to April	Total	16.4 ± 5.0	Weber et al. (2013a, b)
Leporinus taeniatus (Lütken, 1875)	São Francisco	November to February	Total	17.7 ± 1.3	Arantes et al. (2017), Thomé et al. (2005)
Moenkhausia costae (Steindachner, 1907)	São Francisco	Whole year	Multiple	4.0 ± 2.1	Bazzoli et al. (1997)
Prochilodus argenteus (Spix and Agassiz, 1829)	São Francisco	November to January	Total	14.3 ± 3.4	Sato et al. (2005), Arantes et al. (2013)
Prochilodus costatus (Valenciennes, 1850)	São Francisco	November to January	Total	8.4 ± 5.8	Arantes et al. (2013)
Prochilodus lineatus (Valenciennes, 1837)	Grande	November to February	Total	10.1 ± 4.6	Perini et al. (2013)
Pygocentrus piraya (Cuvier, 1819)	São Francisco	January to April	Multiple	5.8 ± 1.8	Ferreira et al. (1996)
Roeboides xenodon (Reinhardt, 1851)	São Francisco	Whole year	Multiple	4.0 ± 3.9	Bazzoli et al. (1997)

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TABLE 13.3 Breeding season, spawning type and gonadosomatic index (GSI, mean ± DP) of ripe females in fish from the Grande and São Francisco Rivers basins, Brazil.—cont'd

Species/Authority	River basin	Breeding season	Spawning	GSI	References
Salminus franciscanus (Lima and Britski, 2007)	São Francisco	November to February	Total	11.2 ± 1.6	Freitas et al. (2013)
Schizodon knerii (Steindachner, 1875)	São Francisco	October to April	Multiple	4.7 ± 1.0	Brandão et al. (2017)
Schizodon nasutus (Kner, 1858)	São Francisco	September to April	Multiple	8.3 ± 3.5	This chapter
Serrasalmus brandtii (Lütken, 1875)	São Francisco	September to June	Multiple	5.4 ± 1.2	Honorato-Sampaio et al. (2009)
Serrasalmus spilopleura (Kner, 1858)	Grande	Whole year	Multiple	4.9 ± 1.1	Lamas and Godinho (1996)
Steindachnerina insculpta (Fernández-Yépez, 1948)	Grande	September to March	Multiple	13.3 ± 5.1	Ribeiro et al. (2007)
Siluriformes					
Conorhynchus conirostris (Valenciennes, 1840)	São Francisco	December to March	Total	5.5 ± 3.3	This chapter
Iheringichthys labrosus (Lütken, 1874)	Grande	October to May	Multiple	4.6 ± 2.2	Santos et al. (2004)
Lophiosilurus alexandri (Steindachner, 1876)	São Francisco	October to February	Multiple	2.0 ± 0.4	Barros et al. (2007)
Pimelodus maculatus (Lacepède, 1803)	São Francisco	September to March	Multiple	7.3 ± 0.6	This chapter
Pseudoplatystoma corruscans (Spix and Agassiz, 1829)	São Francisco	October to February	Total	4.6 ± 2.1	Brito and Bazzoli (2003)
Perciformes					
Cichla kelberi (Kullander and Ferreira, 2006)	São Francisco	Whole year	Multiple	2.7 ± 0.9	Normando et al. (2009)
Cichla piquiti (Kullander and Ferreira, 2006)	Grande	Whole year	Multiple	1.6 ± 0.8	Vieira et al. (2009)

CFI, which generally peaks at early ripening, as was detected in the dogfish *Galeocharax knerii* from the Furnas and Itumbiara reservoirs (Magalhães et al., 2004). However, the CFI and K values may show different patterns with high values related to nutrient abundance in environments with high levels of productivity, such as in river stretches with domestic sewage discharge, as detected for *Astyanax fasciatus* in the Furnas reservoir, Grande River basin (Prado et al., 2011), and *Astyanax rivularis* in the das Velhas River, São Francisco River basin (Weber et al., 2017). The stomach repletion index (SRI = 100 SW/BW; SW, stomach weight) usually follows the same trend as the K during the reproductive cycle.

Endocrine disruption

When environmental conditions are altered due to exposure to estrogenic endocrine disrupting compounds (EDCs), the hepatocytes of males produce Vtg and Zrp in increased amounts similar to levels found in females. EDCs are exogenous substances such as natural and synthetic hormones, plasticizers such as bisphenol A and phthalates, pharmaceuticals such as ethinylestradiol, organochlorine pesticides, and heavy metals released into the aquatic environment by domestic and industrial effluents and by agriculture. They can interfere with the regulation of the endocrine system of organisms, affecting the health of animals and humans alike. EDCs can either mimic endogenous hormones or alter the hormone homeostasis,

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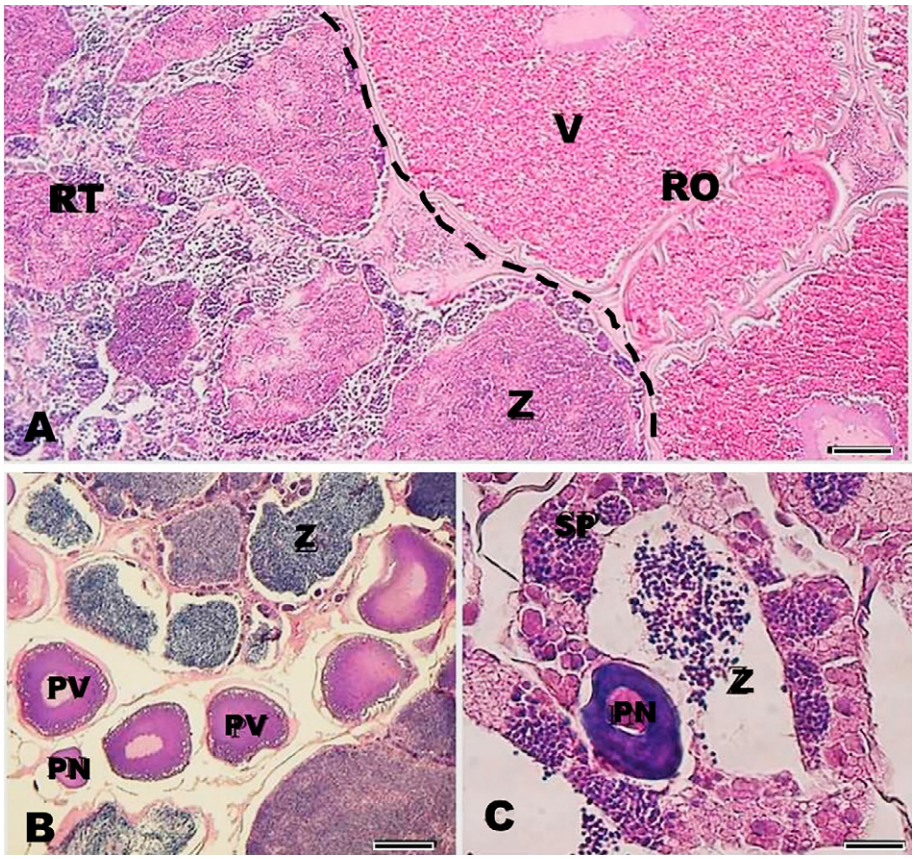

FIG. 13.5 Histological sections of intersex gonad in lambari *Astyanax fasciatus*. (A) Focal arrangement of the ovarian tissue (RO) showing vitellogenic follicles (V) associated with testicular tissue (RT) in different foci, and normal development of the germ cells in the seminiferous tubules and spermatozoa (Z) into tubular lumens. (B–C) Multifocal arrangement with perinucleolar follicles (PN) and previtellogenic follicles (PV) associated with spermatocytes cysts (SP) in the seminiferous tubules. Bars: (A) 112 μm, (B) 167 μm, (C) 125 μm. *Source: Prado, P.S., Souza, C.C., Bazzoli, N., Rizzo, E., 2011. Reproductive disruption in lambari Astyanax fasciatus from a Southeastern Brazilian reservoir. Ecotoxicol. Environ. Saf. 74, 1879–1887.*

interfering with the synthesis, metabolism, and excretion of endogenous hormones (Denslow and Sepúlveda, 2007). The key biomarkers of endocrine disruption are Vtg and Zrp, but insulin-like growth factors (IGF I and II) and estrogen receptors (ERα and ERβ) are also altered by estrogenic contamination (Prado et al., 2014, 2018; Weber et al., 2018). Changes in gonadal development and maturation, fecundity, egg quality, and sex ratio as well as the presence of intersex fish were detected in species of the *Astyanax* genus, which are used as bioindicators of EDC contamination in southeastern Brazil (Prado et al., 2011, 2014; Weber et al., 2017). Two patterns of ovarian follicle organization can be found in the testes of intersex fish: (1) focal arrangement (Fig. 13.5A) with large groups of perinucleolar and vitellogenic follicles associated with testicular tissue in different foci; and (2) multifocal arrangement (Fig. 13.5B–C) with individualized perinucleolar follicles or small groups within the testicular tissue. The focal arrangement occupies a considerable space in the male gonad and may disorganize the testis structure, which was the predominant pattern in the *A. fasciatus* intersex found close to domestic sewage disposal sites in the Furnas reservoir (Prado et al., 2011). The multifocal arrangement was found in *Astyanax rivularis* in streams contaminated by EDCs in the Upper das Velhas River (Weber et al., 2017).

Ovulation and spawning

After maturation, oocytes are released from the ovarian follicles in the ovigerous lamellae during ovulation, and are later released into the external environment in spawning. In cystovarian-type ovaries (Hoar, 1969), including those from most neotropical freshwater teleost fish, oocytes are released from the ovigerous lamellae within the ovarian lumen and carried to the external environment through the ovarian duct. In gymnovarian-type ovaries (Hoar, 1969), the ovarian lamellae open and the oocytes are released directly into the coelomic cavity, such as found in salmon (*Oncorhynchus* and *Salmo* genera).

In tropical environments, two types of spawning—total and multiple or fractioned—are well established. Total spawning is associated with sites with significant seasonal variations, that is, lotic environments, whereas multiple spawning occurs more frequently in lentic environments such as river backwaters, lakes, weirs, and reservoirs (Godinho et al., 2010). Multiple spawning indicates that fish spawn several times in the same breeding season, and therefore this allows asynchronous development of eggs and larvae, which may consequently occupy distinct niches. This leads to

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less competition among adults for spawning grounds and among larvae for food sources (Nikolsky, 1963). Total spawning is characteristic of the *piracema* fish species, which performs reproductive migration to spawn in the headwaters of rivers, similar to curimatãs *P. argentus* and *P. costatus*, dourado *S. franciscanus*, surubim *P. corruscans*, and others (Sato et al., 2003b). Fish with multiple spawnings, such as the piranhas *P. piraya*, pirambeba *S. brandtii*, traira *Hoplias malabaricus*, and trairão *H. lacerdae,* have a long reproductive period while those of total spawning have a short reproductive period (Sato et al., 2003b).

Several criteria are important in determining the type of spawning of a fish species: oocyte development type, modal distribution of vitellogenic follicles diameters, histological characteristics of the spawned ovaries, variations of GSI, and frequencies of the gonadal maturation stages (Bazzoli, 2003). In general, total spawning species present group-synchronous oocyte development, a unimodal distribution of vitellogenic follicles, a short spawning season, and an abrupt reduction of GSI after spawning. On the other hand, multiple spawner fish have asynchronous oocyte development, two or more lots of vitellogenic follicles, oocytes are released in batches in a prolonged spawning season, a slow and progressive decrease of GSI, and a high frequency of partially spawned females.

In total spawning fish, histological sections of mature ovaries show abundant perinucleolar and vitellogenic follicles, which is a characteristic of group-synchronous oocyte development in two groups, with perinucleolar follicles being the reserve stock for the next reproductive cycle (Fig. 13.2C). In multiple spawning species, histological sections of ripe ovaries present follicles at different development stages. Partially spawned ovaries present postovulatory follicles alongside vitellogenic, previtellogenic, perinucleolar, and atretic follicles (Fig. 13.2E). Totally spawned ovaries present perinucleolar follicles, postovulatory follicles, and eventually vitellogenic follicles in atresia (Fig. 13.2F).

Ovarian remodeling after spawning

When females of migratory species are kept in captivity and submitted to hypophysation, their ovaries present numerous postovulatory follicles (POFs) and atretic follicles after spawning (Fig. 13.6); this is a good model for studying post-spawning ovarian remodeling. In the POFs, the follicular lumen is surrounded by follicular cells attached to the basement membrane and connective theca externally. In fish that exhibit external fertilization, POFs do not present hormonal activity and, unlike mammals, they do not develop a corpus luteum. During POF remodeling, follicular cells are progressively eliminated by programmed cell death by apoptosis, as detected by transmission electronic microscopy, electrophoresis in agarose gel, a TUNEL assay, and an immunohistochemical reaction for caspase-3, an effector molecule of apoptosis (Fig. 13.7A–E). In spawned ovaries, POFs are quickly reabsorbed, that is, approximately 5–7 days after spawning (Drummond et al., 2000; Santos et al., 2005, 2008a; Thomé et al., 2006).

When females did not spawn in captivity, an intense process of follicular atresia developed in their ovaries, and this led to the progressive resorption of nonovulated oocytes. Follicular atresia can be induced by a variety of endogenous and exogenous factors, including the reduction of hormone levels, nutrient deprivation, and toxic agents present in the environment. This process mainly affects vitellogenic follicles and may reduce the reproductive potential of the species (Rizzo and Bazzoli, 1995). Several events characterize follicular atresia in fish ovaries: nuclear envelope rupture, zona radiata splits, and yolk degradation and liquefaction followed by phagocytosis by hypertrophied follicular cells, progressive reduction of follicle size, and theca thickening (Santos et al., 2008b). The end of follicular atresia is characterized by

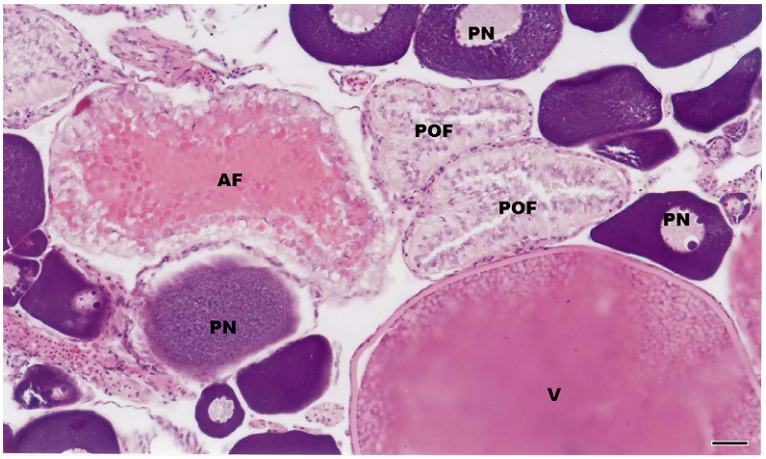

FIG. 13.6 Histological section of ovary in the lambari *Astyanax bimaculatus* after spawning showing perinuleolar (PN), vitellogenic (V), postovulatory (POF), and atretic (AF) follicles. Bar: 95 μm.

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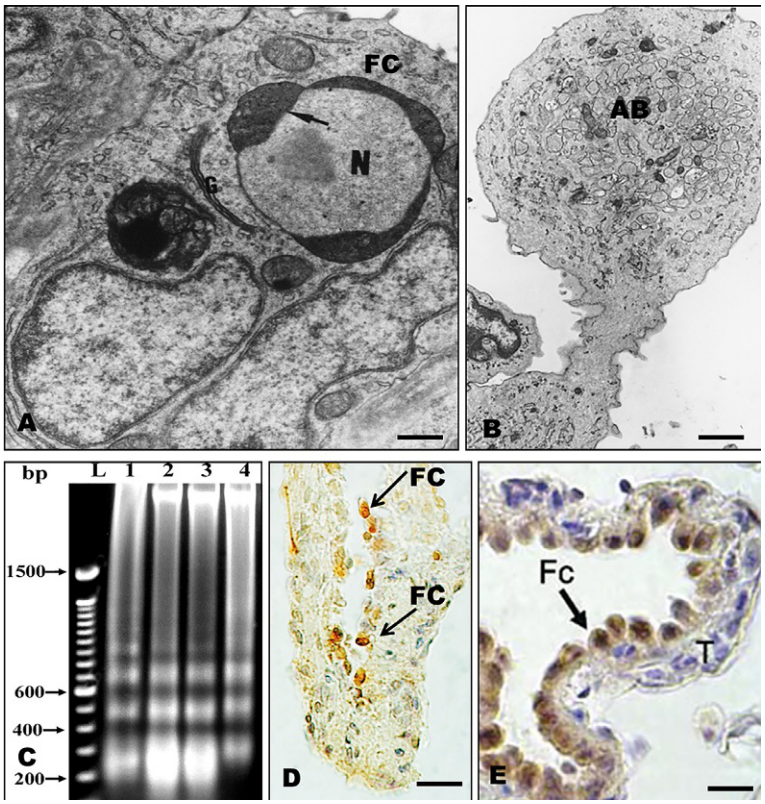

FIG. 13.7 Apoptosis during postovulatory follicle remodeling in (A, B) lambari *Astyanax bimaculatus*, (D) piau *Leporinus taeniatus*, and (C, E) curimatã *Prochilodus argenteus*. (A) Electron microscopy of the follicular cell (FC) in apoptosis, showing evident pattern of condensation chromatin close the nuclear envelope (arrow). (B) Ultrastructure of the formation of the apoptotic body (AB). (C) Agarose gel electrophoresis of apoptotic DNA: (L) standard DNA ladder, (lane 1) positive control, DNA extracted of rat thymus, (lanes 2–5) DNA extracted of spawned ovary at 0, 3, and 5 days after spawning, respectively. (D) TUNEL reaction in the follicular cells (FC) during regression of the postovulatory follicle. (E) Immunohistochemical reaction for caspase-3 in follicular cells. Bars: (A) 1.6 μm, (B) 2.5 μm, (D) 1.8 μm, (E) 12.7 μm. *Source: (A, B) Drummond, C.D., Bazzoli, N., Rizzo, E., Sato, Y., 2000. Postovulatory follicle: a model for experimental studies of programmed cell death or apoptosis in teleosts. J. Exp. Zool. 287, 176–182, (C, E) Santos, H.B., Thomé, R.G., Arantes, F.P., Sato, Y., Bazzoli, N., Rizzo, E., 2008b. Ovarian follicular atresia is mediated by heterophagy, autophagy, and apoptosis in Prochilodus argenteus and Leporinus taeniatus (Teleostei: Characiformes). Theriogenology 70, 1449-1460, (D) Santos, H.B., Rizzo, E., Bazzoli, N., Sato, Y., Moro, L., 2005. Ovarian regression and apoptosis in the South American teleost Leporinus taeniatus Lütken (Characiformes, Anostomidae) from the São Francisco Basin. J. Fish Biol. 67, 1446–1459.*

the formation of yellow bodies, consisting of agglomerated phagocytic cells that accumulate brownish pigments associated with blood vessels and connective fibers (Miranda et al., 1999). These structures have no endocrine activity and are similar to melano-macrophage centers, which are aggregated with macrophages with pigments of hemosiderin, melanin, and lipofuscin frequently found in the spleen, kidney, and liver (Ribeiro et al., 2011).

The remodeling of the nonspawned ovaries is a long process, varying from 4 to 7 months, depending on the species and the stocking conditions (Miranda et al., 1999). Studies on two captive fish species (Santos et al., 2008b; Thomé et al., 2009) showed that follicular atresia is mediated by phagocytosis, autophagy, and apoptosis (Fig. 13.8A–C). Moreover, an interplay between autophagy and apoptosis was also found in follicular cells during POF resorption (Morais et al., 2016; Thomé et al., 2009). These data indicate that different signaling pathways contribute to ovarian regression after spawning. In the Nile tilapia, the frequency of mature vitellogenic follicles significantly increases during ovarian recrudescence after spawning, and reaches 35% at 21 days postspawning at temperatures ranging from 28°C to 30°C (Melo et al., 2014). As this species presents multiple spawning and a short interval between spawns, follicular development occurs simultaneously with the resorption of POFs and atretic follicles. Therefore, ovarian remodeling in the Nile tilapia is faster than in species with total spawning. In this species, apoptosis was observed at the beginning of POF regression and at the end of follicular atresia while an immunohistochemical reaction for the proliferating cell nuclear antigen (PCNA) indicated proliferation of theca cells at the end of atresia (Melo et al., 2015).

Reproductive period and spawning season

Environmental stimuli such as temperature, photoperiod, and rainfall are critical in triggering gametogenesis and are responsible for determining gonadal maturity and the fish spawning season (Lowe McConnel, 1987). When environmental conditions are suitable in the breeding season, food availability increases, favoring the survival and growth of the offspring. In temperate regions, seasonal variations of environmental parameters are well defined, along with the reproductive cycle of fish. A long-term breeding season is common in tropical regions, but seasonal changes in reproductive activity are found mainly in *piracema* species. Rainfall triggers spawning, and the increased water flow in the river is important to carry eggs

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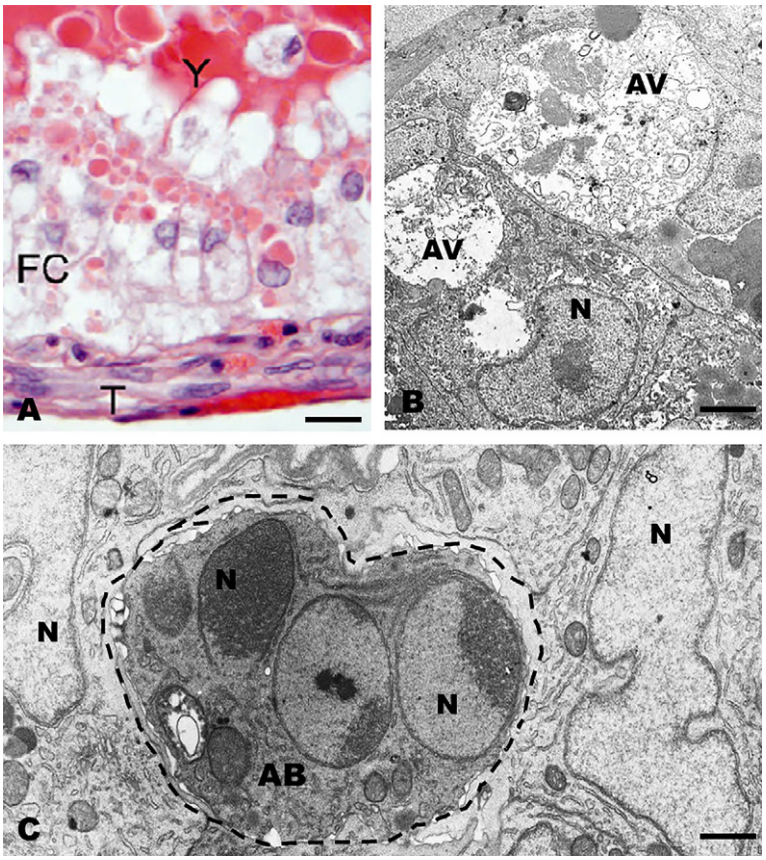

FIG. 13.8 (A) Histology and (B, C) electron microscopy of the follicular atresia in ovary of curimatã *Prochilodus argenteus*. (A) Phagocytosis, (B) Autophagy, and (C) Apoptosis interplay during degeneration of the follicular cells. Yolk (Y) phagocytosis by follicular cells (FC), large autophagic vacuole (AV) with mitochondria in degeneration, apoptotic body (AB) with three apoptotic nuclei (N) phagocytosed by a follicular cell. Bars: (A) 19.2 μm, (B) 2.26 μm, (C) 1.92 μm. *Source: (A–B) Santos, H.B., Sato, Y., Moro, L., Bazzoli, N., Rizzo, E., 2008a. Relationship among follicular apoptosis, integrin β1 and collagen type IV during early ovarian regression in the teleost Prochilodus argenteus after induced spawning. Cell Tissue Res. 332, 159–170.*

to places that are favorable for the development of embryos and larvae. Moreover, increased oxygenation of the surface water also favors the development of eggs and larvae. Some fish species present reproductive plasticity, and the spawning season may change according to the physiological and environmental conditions. For example, the white piau *S. knerii* spawns throughout the year in the Três Marias reservoir while in the São Francisco River, it only spawns from October to March (Brandão et al., 2017).

In southeastern Brazil, most fish species reproduce between spring and summer (September to February), which corresponds to the period of increasing temperature and rainfall. An analysis of the frequencies of the reproductive cycle stages shows a decrease or even interruption of reproduction in the winter months (June to August), which is considered a resting period (Fig. 13.9). In general, gametogenesis and gonadal maturation begin in the spring (September to November), following an increase in temperature. The late ripening and ripe stages are frequently found in October and November, and the spawning season occurs in the summer (December to February), when the rains are intense. The postspawning period occurs in the autumn (March to May) when the gonads undergo remodeling to return to the resting stage to start a new reproductive cycle. This breeding pattern is common for large migratory species (*piracema* species) such as *P. argenteus* (Sato et al., 2005), *P. lineatus* (Perini et al., 2013), and *S. franciscanus* (Freitas et al., 2013) that reproduce in rivers. In fish that inhabit lentic water, such as those that reproduce in reservoirs, the phases of the reproductive cycle are not well defined and prolonged periods of reproductive activity and spawning are frequently found (Bazzoli and Godinho, 1991; Ribeiro et al., 2007). However, a breeding peak has been associated with the rainy season in some multiple spawning species (Alvarenga et al., 2006; Carvalho et al., 2009; Honorato-Sampaio et al., 2009; Normando et al., 2009; Santos et al., 2004). Continuous gonadal maturation throughout the year and the absence of resting and fully spawned stages were reported for the pirambeba *S. spilopleura* in the Itumbiara reservoir, Paranaíba River, Paraná River basin (Lamas and Godinho, 1996). In the headwaters of the das Velhas River in the São Francisco River basin, the lambari *Astyanax rivularis* shows a prolonged breeding season, late ripening/ripe stages throughout the year with a peak from June to September, and multiple spawning from September and March in the rainy season (Weber et al., 2017). The breeding season and spawning type of fish species from the São Francisco and Grande River basins are found in Table 13.3.

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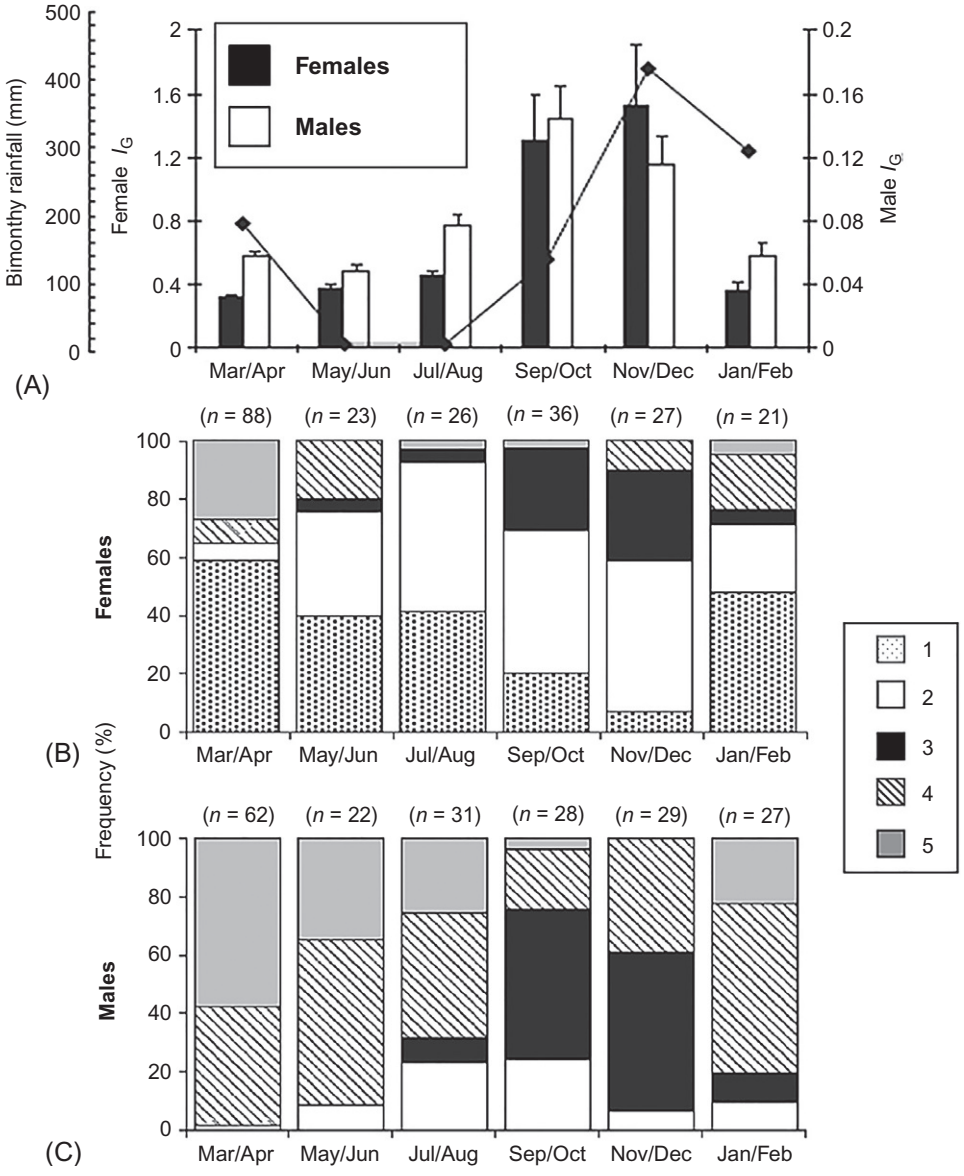

FIG. 13.9 (A) Rainfall accumulated bimonthly and gonadosomatic index (IG), and (B, C) frequency (%) of the reproductive cycle stages in (B) females and (C) males of pirambeba *Serrasalmus brandtii* in the Juramento Reservoir, Upper São Francisco River basin, southeastern Brazil. (1) resting, (2) early ripening, (3) late ripening/ripe, (4A) partially spawned/spent, (4B) totally spawned/spent. *Source: Honorato-Sampaio, K., Santos, G.B., Bazzoli, N., Rizzo, E., 2009. Observations on the seasonal breeding biology and fine structure of the egg surface in the white piranha Serrasalmus brandtii from the São Francisco River basin, Brazil. J. Fish Biol. 75, 1874–1882.*

Fecundity

Fecundity is a measure of the reproductive potential of a species through the number of vitellogenic oocytes present in the ovaries before the breeding season (Bagenal and Braum, 1978). It is an important ecological parameter in studies of population dynamics and allows for reproductive prognoses as well as the establishment of criteria for the exploration and management of commercially important fish species. Species with free eggs have high fecundity to compensate for their loss due to dispersion to unsuitable sites for development (Nikolsky, 1963). On the other hand, fish with adhesive eggs have lower fecundity because there is less egg loss, especially in species that exhibit parental care behavior. For multiple spawning species, fecundity estimates the number of oocytes released in each lot, and it is difficult to determine because the number of vitellogenic oocytes can vary throughout the year depending on the intensity and duration of the rainfall.

Fecundity and egg production are affected by several factors, including environmental variations, food availability, breeding season duration, and spawning frequencies (Vazzoler, 1996). Furthermore, fecundity also increases with fish

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age and size, but the number of oocytes produced decreases in aging fish (Nikolsky, 1963). Regarding exotic species, fecundity increases when they are introduced into environments with more favorable ecological conditions, with negative impact for the native species due to competition for food and spawning grounds (Munthali and Ribbink, 1998). On the other hand, fecundity is reduced under unfavorable conditions, such as in the stretch immediately downstream from the Três Marias dam, São Francisco River (Arantes et al., 2010; Normando et al., 2014; Weber et al., 2013a, b).

Various types of fecundity can be estimated with absolute and relative fecundities being the most studied in natural environments. Total or absolute fecundity evaluates the total number of oocytes that a female produces in a breeding season. It is determined by the count of vitellogenic oocytes present in the ovaries before spawning in the total spawning species. Relative fecundity relates the number of vitellogenic oocytes per weight unit or body length, thus excluding the interference of the fish size. In fish subjected to hormonally induced spawning, fecundity is determined by the number of oocytes released after stripping (Arantes et al., 2013; Sato et al., 2003a). Batch fecundity is estimated when the species has multiple spawnings such as the lambaris *Astyanax* sp. (Prado et al., 2011; Weber et al., 2017). For the commercially important fish species of the São Francisco River basin, high fecundity is characteristic of total spawning fish that have small eggs while multiple spawning fish have lower fecundity (Arantes et al., 2013). Fish with parental care behavior and large oocytes such as the pacamã *Lophisilurus alexandri* have the lowest fecundity values. The spawning type and fecundity of fish species from the São Francisco River are shown in Table 13.4.

Direct and total oocyte counting is the most recommended method for determining fecundity. However, it is extremely labor intensive, especially when the number of oocytes is large; in this case, an automatic counter is recommended. When oocyte distribution is homogeneous throughout the ovaries, samples can be taken to facilitate counting and reduce counting time. This is done by obtaining the total weight of the ovaries, and then three samples of about 1 g are taken from each fish. From the number of oocytes per gram of ovary, the number of oocytes is calculated for the total weight of the ovaries by a simple mathematical rule of three. This method based on ovarian weight (gravimetric method) is the most widely used method to determine fish fecundity (Bagenal and Braum, 1978). Dissociation of oocytes from the sample is generally carried out using Gilson's solution (100 mL of 60% ethanol, 880 mL distilled water, 15 mL of 80% nitric acid, 18 mL glacial acetic acid, and 20 g of mercuric chloride), and the vitellogenic oocytes are separated by size and manually counted (Weber et al., 2017). Alternatively, these counts can be performed directly on mature ovaries, in which vitellogenic oocytes are separated from the oocyte pool by color and size under a stereomicroscope (Prado et al., 2014). In addition to reducing costs and time, the great advantage of this latter method is to avoid the use of Gilson's solution, which contains mercury, a highly toxic metal that can contaminate the environment.

TABLE 13.4 Spawning type and fecundity (mean ± DP) of fish species from the São Francisco River, Brazil.

Species/Authority	Spawning	AF ($\times 10^3$)	RF/g BW	References
Characiformes				
Acestrorhynchus lacustris (Lütken, 1875)	Multiple	9.0 ± 6.2	64.7 ± 16.2	This chapter
Astyanax fasciatus (Cuvier, 1819)	Multiple	38.2 ± 2.2	983.8 ± 232.1	Carvalho et al. (2009), Prado et al. (2011)
Astyanax rivularis (Lütken, 1875)	Multiple	25.9 ± 10.8	952.9 ± 161.2	Weber et al. (2017)
Bryconops affinis (Günther, 1864)	Multiple	2.8 ± 1.1	127.0 ± 44.0	This chapter
Brycon orthotaenia (Günther, 1864)	Total	337.6 ± 152.5	183.0 ± 23.1	Arantes et al. (2013)
Curimatella lepidura (Eigenmann and Eigenmann, 1889)	Total	40.9 ± 8.8	863.0 ± 91.0	Alvarenga et al. (2006), Perini et al. (2013)
Leporinus obtusidens (Valenciennes, 1837)	Total	538.0 ± 223.1	524.1 ± 89.8	Arantes et al. (2013)
Leporinus piau (Fowler, 1941)	Total	68.3 ± 24.9	355.6 ± 56.4	This chapter
Leporinus reinhardti (Lütken, 1875)	Total	73. 37 ± 33.21	295.2 ± 61.1	This chapter
Orthopinus franciscensis (Eigenmann, 1914)	Multiple	1.9 ± 0.5	294.0 ± 84.0	This chapter
Prochilodus argenteus (Spix and Agassiz, 1829)	Total	397.5 ± 206.9	236.6 ± 44.5	Arantes et al. (2013)
Prochilodus costatus (Valenciennes, 1850)	Total	251.9 ± 173.6	324.5 ± 48.2	Arantes et al. (2013)

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TABLE 13.4 Spawning type and fecundity (mean ± DP) of fish species from the São Francisco River, Brazil.—cont'd

Species/Authority	Spawning	AF ($\times 10^3$)	RF/g BW	References
Salminus franciscanus (Lima and Britski, 2007)	Total	583.9 ± 339.8	145.9 ± 18.7	Freitas et al. (2013)
Schizodon knerii (Steindachner, 1875)	Multiple	87.1 ± 20.7	170.7 ± 35.5	Brandão et al. (2017)
Steindachnerina elegans (Steindachner, 1875)	Multiple	22. 1 ± 8.7	810.0 ± 148.0	Perini et al. (2013)
Tetragonopterus chalceus (Spix and Agassiz, 1829)	Multiple	8.4 ± 3.9	400.0 ± 148.0	This chapter
Triporteus guentheri (Garman, 1890)	Multiple	2.3 ± 1.0	79.0 ± 25.0	This chapter
Siluriformes				
Lophisilurus alexandri (Steindachner, 1876)	Multiple	4.5 ± 1.3	1.4 ± 0.3	This chapter
Pimelodus maculatus (Lacepède, 1803)	Multiple	91.8 ± 48.7	180.8 ± 25.7	Arantes et al. (2013)
Pseudoplatystoma corruscans (Spix and Agassiz, 1829)	Total	1329.4 ± 527.4	113.7 ± 21.4	Arantes et al. (2013)
Rhinelepis aspera (Spix and Agassiz, 1829)	Multiple	149.5 ± 68.5	98.0 ± 15.0	This chapter
Perciformes				
Cichla kelberi (Kullander and Ferreira, 2006)	Multiple	8.1 ± 2.7	10.6 ± 2.1	Normando et al. (2009)

AF, absolute fecundity, that is, number of ripe oocytes in the ovaries or oocytes released in the induced spawning; *RF/g BW*, relative fecundity, that is, number of ripe oocytes/g of body weight.

Types of eggs and fertilization

Fish eggs have macroscopic and microscopic characteristics that vary according to the reproductive strategies of the species. In ichthyoplankton samples, the identification of fish eggs can provide information on spawning areas and the biology of their dispersion. The macroscopic criteria such as diameter, color, degree of adhesiveness, chorion thickness, and presence or absence of gelatinous coating are not very accurate for identifying species or even the genus to which they belong.

Fish eggs may be pelagic or demersal according to their specific gravity. Pelagic eggs are common in marine species and have one or more features that allow their flotation (Braum, 1978): one or more oil droplets in the yolk, high water content in the yolk, increased egg diameter due to the presence of a gelatinous layer, and increased perivitelline space. Freshwater teleost fish eggs are usually demersal, that is, they have a specific gravity greater than that of water. They can be deposited on the bottom of rivers or reservoirs and adhere to some type of substrate, or they remain in the water column for dispersion. In captivity, eggs of commercially important species such as curimatãs *P. costatus* and *P. argenteus*, dourados *S. brasiliensis* and *S. franciscanus*, surubim catfish *P. corruscans*, and piaus *L. piau*, *L. reinhardtii*, and *S. knerii* are heavier than water, that is, demersal, and remain in the water column of the incubators during development. However, when the water flow is cut, they are deposited on the bottom of the incubators.

In relation to adhesiveness, freshwater fish eggs can be free (nonadhesive) or they may present different adhesiveness degrees (Godinho et al., 2010; Rizzo et al., 2002). In general, the eggs of commercially important migratory species are free and numerous with a small diameter and a wide perivitelline space, characteristics that facilitate their dispersion to favorable places for development. Species that do not need to carry out long reproductive migrations to spawn, in general, reproduce in reservoirs and streams. Their eggs are less numerous as well as being free or with some degree of adhesiveness and with varying diameters and small perivitelline spaces; also, these species may present parental care behavior (Sato et al., 2003b). With a scanning electron microscope, Characiformes oocytes show structural variations in the organization of the oocyte's surface related to the egg adhesiveness and to the spawning substrate (Rizzo et al., 2002). Thus, free or nonadhesive eggs may have a smooth surface with pore canals or a thin fibril network covering the zona radiata while

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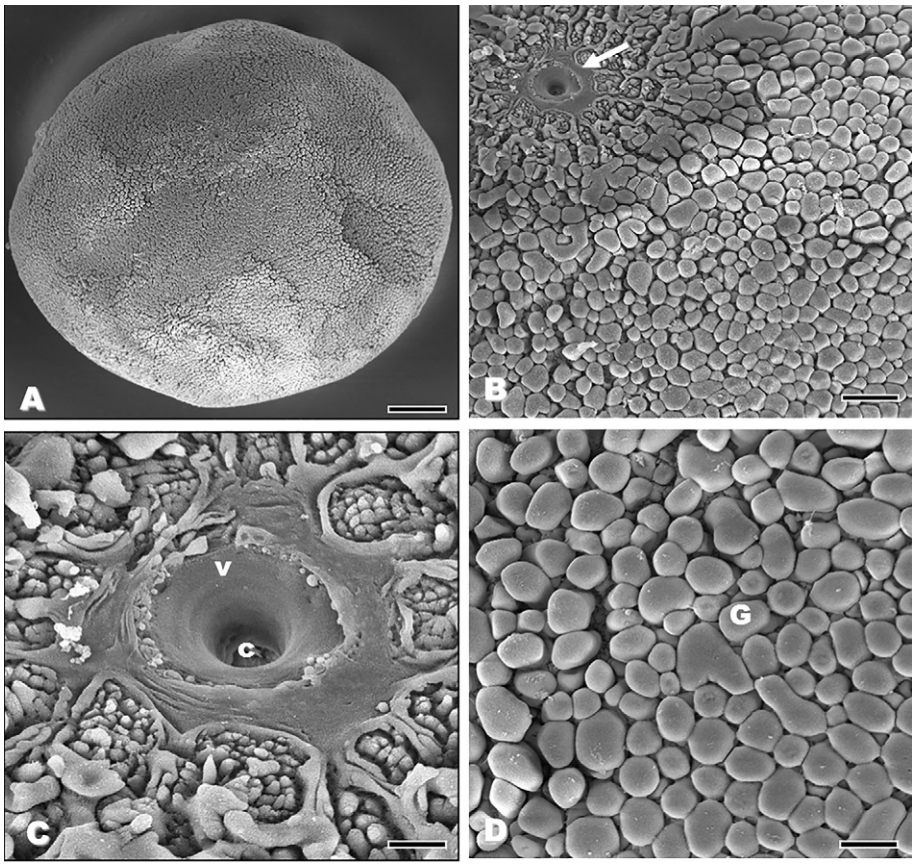

FIG. 13.10 Scanning electron microscopy of adhesive oocyte in the white piau *Schizodon knerii*. (A) Recently spawned oocyte. (B) Animal pole surface where the micropyle is placed (arrow). (C) Micropyle with a vestibule (v) and a micropylar canal (c) surrounded by globules. (D) Vegetal pole coated by adhesive globules. Bars: (A) 104.2 μm; (B) 23 μm; (C, D) 5.7 μm.

adhesive eggs have surface specializations varying in shape and size associated with the zona radiata: filaments, globules, and villus (Rizzo et al., 2002). In the white piau *Schizodon knerii,* spawned oocytes have globules covering the zona radiata in both animal and vegetal poles (Fig. 13.10A–D). In Siluriformes, both nonadhesive (free) and adhesive eggs are covered by a gelatinous coating (jelly coat) such as in the catfish *Paulicea lutkeni* and *Rhinelepis aspera*, respectively (Fig. 13.11A–D).

After spawning, the recently spawned oocytes of most freshwater fish exhibit intact yolk globules without fusion or liquefaction, cortical alveoli in the peripheral ooplasm, thick zona radiata with transversal striations, and basophilic granules that converge toward the micropyle at the animal pole (Fig. 13.12A–E). Through the micropyle, the fertilizing spermatozoon has direct access to the oocyte membrane, without acrosome reaction. In most species, the micropyle has a vestibule and a micropylar canal, which houses a modified follicular cell called the micropylar cell, during oocyte development (Figs. 13.10C and 13.11A and C). The set of micropyle and micropylar cells constitutes the micropylar apparatus, whose morphology is similar in species belonging to the same systematic group (Ricardo et al., 1996). With the scanning electron microscope, the surface of the micropylar region shows species-specific ultrastructural features, such as the presence of folds or ridges of the zona radiata in the lambari *Astyanax bimaculatus* or knobs in *Pimelodus maculatus*, that may be important in fertilization (Rizzo et al., 2002). At the base of the micropyle of several fish species, the plasma membrane presents a distinct area with microvilli that wrap the sperm during fertilization (Hart, 1990).

In fish with external fertilization, the interaction between gametes depends on the presence of water because the sperm acquire motility and the oocytes are activated to complete the second meiotic division, blocked in metaphase II. These events lead to the elimination of the second polar body, the formation of the male and female pronuclei, the fusion of the genetic material, and the formation of the zygote. After freshwater fish oocytes are released into water, their fertility is limited to a few seconds after ovulation. When spermatozoa lose their motility or when the micropylar canal closes, fertilization does not occur. Then, eggs divide by parthenogenesis but gradually lose viability and degenerate during embryonic development (Rizzo et al., 2003).

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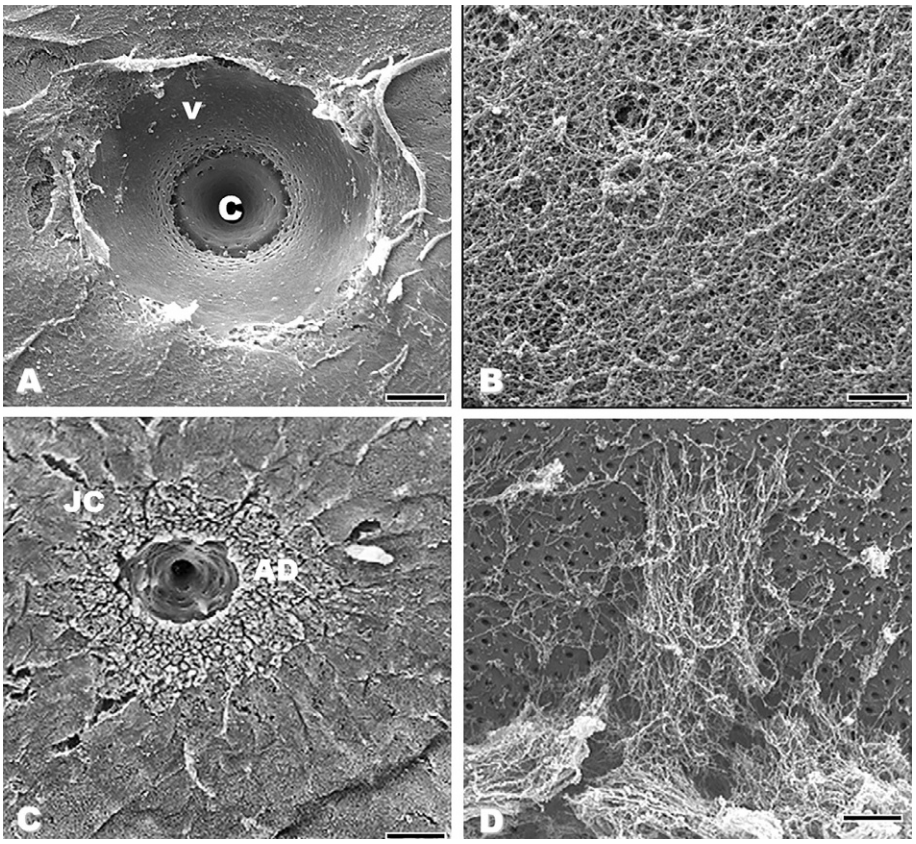

FIG. 13.11 Scanning electron microscopy of (A–B) nonadhesive oocyte in catfish *Paulicea lutken* and (C–D) adhesive oocyte of the black catfish *Rhinelps aspera* released after induced spawning. (A) Micropyle with a vestibule (v) and a micropylar canal (c). (B) Fibrous layer (jelly coat) is concentrated at the animal pole. (C) Micropyle surrounded by an adhesive disc (AD) and jelly coat (JC). (D) Pore canals of the zona radiata covered by residues of the jelly coat, which was extracted in the vegetal pole. Bars: (A) 5.8 μm; (B, D) 2.3 μm; (C) 11.4 μm.

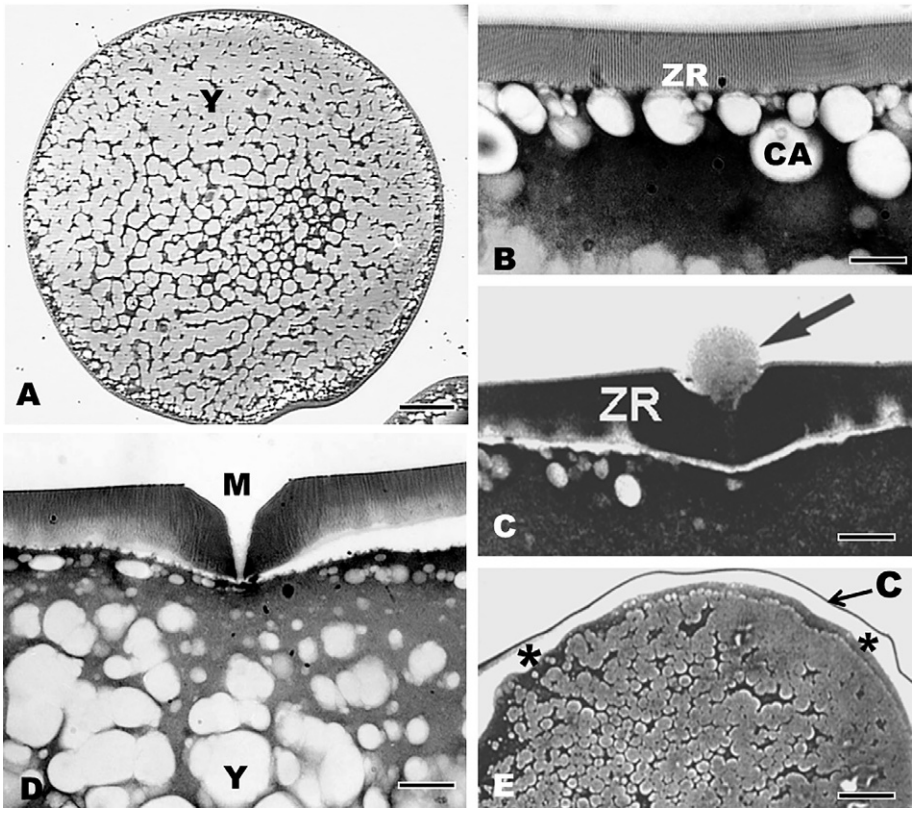

FIG. 13.12 Oocytes of curimatã *Prochilodus costatus* released after induced spawning. (A) Ooplasm with whole yolk globules (Y) and basophilic material; (B) Detail of the zona radiata with transversal striations (ZR), cortical alveoli (CA), and yolk globules (Y) at the vegetal pole; (C) Funnel-shaped micropyle; (D) Fertilization cone (arrow) occludes the micropyle during fertilization. (E) Formation of the perivitelline space. Bars: (A) 168 μm, (B, C, D) 7.3 μm, (E) 84.5 μm.

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After gamete fusion, a fertilization cone is formed that is defined as a reaction of the egg cortex to sperm penetration. In this condition, the cortical ooplasm protrudes into the interior of the micropyle, helping the penetration of the fertilizing sperm and expelling the supernumerary. The perivitelline space fluid has a strong agglutinating effect on spermatozoa and forms a buffer that seals the micropylar canal, avoiding polyspermy (Hart, 1990). In eggs of the pacu *P. mesopotamicus*, the fertilization cone was observed 1–2 min after fertilization (Rizzo et al., 1997).

After the activation of the gametes, the cortical reaction occurs, which consists of the exocytosis of the cortical alveoli glycoconjugates, and a perivitelline space is formed between the oocyte membrane and the zona radiata. The high molecular weight of these substances increases the osmotic pressure of the perivitelline fluid leading to egg hydration, an increase in oocyte diameter, and a reduction of zona radiata thickness (Hart, 1990). At the same time, the cortical alveoli glycoconjugates cause physical and chemical changes in the zona radiata, making it rigid and referred to as chorion (Yamagami et al., 1992). The cortical reaction occurs regardless of fertilization and may be induced by the increase of the calcium concentration released from the intracellular stocks and culminates with micropyle closure (Redding and Patino, 1993).

Although the fertilization cone and other factors contribute to blocking polyspermy, monospermic fertilization is guaranteed in teleosts primarily by the micropylar canal diameter, which is similar to the sperm head diameter in most species and restricts the oocyte membrane area exposed to the interaction with spermatozoa (Hart, 1990; Redding and Patino, 1993). The fact that the primary block is due to the micropyle structure itself can be experimentally verified by extracting the zona radiata surrounding the newly fertilized oocyte, in which case the fertilization is polyspermic (Ohta and Nashirozawa, 1996). Cross-fertilization between closely phylogenetic-related species is used in fish farming for producing hybrids more suitable for industrial scale production, commercialization, and consumption. However, species-specific fertilization is guaranteed in nature by the differences in habitats, spawning seasons, gamete morphology, and mating behavior of fish (Ohta, 1985).

Embryonic development

In order to introduce a wild species in fish farming, previous knowledge of the embryogenesis and larval ontogenesis is needed because these are the critical phases that limit the survival of the offspring.

Fish oocytes are being classified as the telolecitic type, that is, they exhibit a large yolk supply to nourish the developing embryo and the larva until the development of the digestive system concomitantly to the mouth opening. Several factors may affect the duration of embryonic development such as food supply, temperature, photoperiod, and other physicochemical and physiological parameters. Rising temperatures speed up embryonic development, chorion rupture, and larva hatching. In southeastern Brazil, temperatures between 24°C and 26°C have been frequently employed in the induced spawning and embryo development of neotropical fish species (Arantes et al., 2011b). Higher temperatures can cause morphological changes in the larvae, decreasing the survival rates of the fingerlings. Hatching time in *Leporinus piau* was 21 h at 24°C or 504 degree hours, that is, water temperature × hatching time (Borçato et al., 2004). Similar values in degree hours were found for other *Leporinus* species at 24ºC (Sato et al., 2003a). On the other hand, the embryonic development of *Leporinus macrocephalus* was 324 degree hours at 28°C (Reynalte-Tataje et al., 2001). However, hatching was more rapid in *A. bimaculatus*, that is, 17 h at 24°C; this is a characid species with an opportunistic reproductive strategy (Weber et al., 2013a, b).

In aquaculture, reproductive efficiency is determined by fertilization, hatching, and larval abnormality rates. High rates of fertilization and hatching and low rates of larval abnormality are important to ensure the quality of the offspring produced in fish farming. When ovulated oocytes of neotropical species are kept in the ovarian cavity prior to spawning (in situ stock) or outside it (ex situ stock) before fertilization, eggs rapidly become less viable and fertilization rates decrease while larval abnormalities increase with increasing stocking time (Rizzo et al., 2003).

Embryonic development begins with fertilization of the oocyte, and the following stages have been considered for most fish species.

Zygote

At this stage, the animal and vegetal poles are segregated to form the blastodisc (Fig. 13.13A). Basophilic granular material concentrates in the animal pole, where the micropyle is located. In the vegetal pole, yolk globules accumulate as a nutrient reserve for embryo development. The exocytosis of the cortical alveoli increases the osmotic pressure and leads to the influx of water, creating a perivitelline space that protects the embryo from environmental aggressions and allows for egg survival in water currents.

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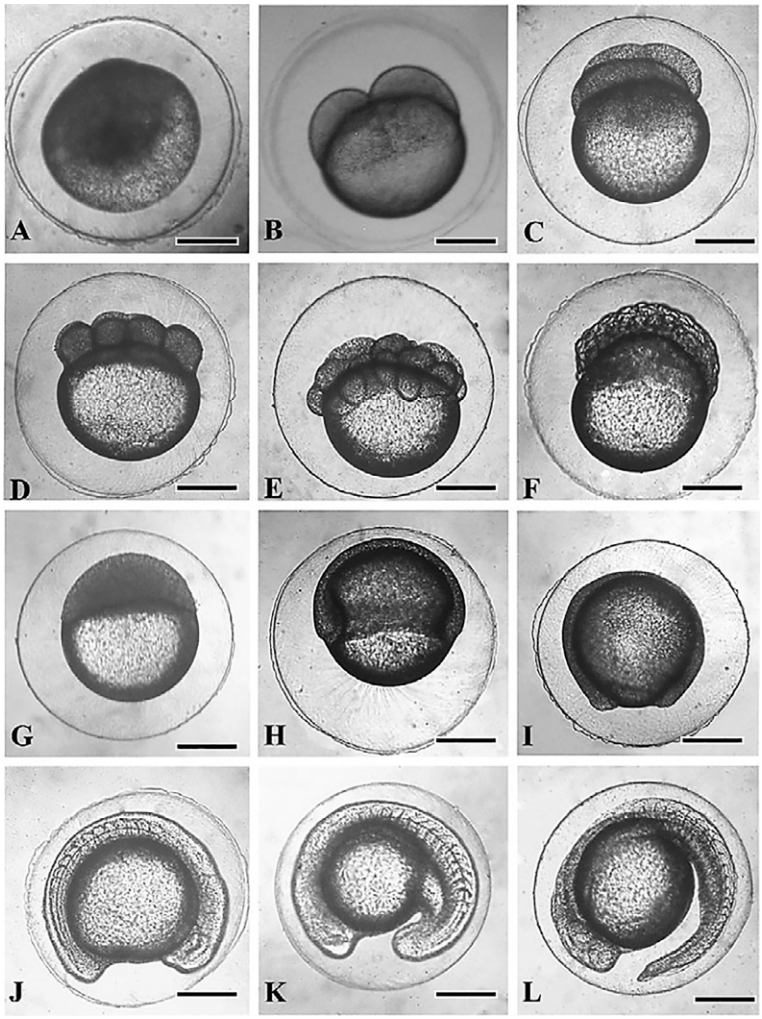

FIG. 13.13 Embryonic development stages in lambari *Astyanax bimaculatus* at 24°C. (A) Zygote, 20 min; (B–F) Cleavage, 40 min; (G) Blastula, 2 h 45 min; (H) Gastrula, 3 h 45 min; (I) Blastopore closure, 5 h; (J) Differentiation of embryonic layers, 7 h 15 min; (K) Somitogenesis and early tail release, 10 h 30 min; (L) Completely free tail at pre-hatching, 17 h. Bars: (A–L) 326 μm. *Source: Weber, A.A., Arantes, F.P., Sato Y., Rizzo E., Bazzoli, N., 2013a. Oocyte adhesiveness and embryonic development of Astyanax bimaculatus (Linnaeus, 1758) (Pisces: Characidae). Zygote 21, 198–202; Weber, A.A., Nunes, D. M. F., Gomes, R. Z., Rizzo, E., Santiago, K.B., Bazzoli, N., 2013b. Downstream impacts of a dam and influence of a tributary on the reproductive success of Leporinus reinhardti in São Francisco River. Aquat. Biol. 19, 195–200.*

Cleavage

Fish egg segmentation is of the meroblastic type and happens in the animal pole (Fig. 13.13B–F). This phase is characterized by successive mitotic divisions of the blastodisc, and then blastomeres are formed. The blastodisc divisions are synchronous and oriented up to 32 or 64 blastomeres, depending on the species. Then, successive divisions form smaller blastomeres.

Blastula

In this phase, successive divisions of the blastodisc form layers of blastomeres that protrude from the animal pole, the high blastula (Fig. 13.13G). Blastomeres divide into several cleavage planes. In general, small cavities are formed between blastomeres, but a typical blastocoel is not formed, a characteristic of the morula of other vertebrates. The blastomeres gradually flatten, and small and ovoid nuclei form the periblast in the border zone between the animal and vegetal poles. The function of the periblast is to direct morphogenetic movements, favoring epiboly during gastrulation. At the end of this phase, the blastomeres located at the margin of the blastodisc join to form the vitelline syncytial layer, which consists of a continuous cytoplasmic strip with several nuclei.

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Gastrula

Embryo genome activation and cell movements occur in this phase, which is characterized by epiboly gradually covering the yolk (Fig. 13.13H). The blastodisc cells move and form the epiblast and the hypoblast. The epiblast and the vitelline syncytial layer expand like a mantle, which initially covers half the yolk (50% epiboly), then 75%, and finally closes the blastopore (100% epiboly). The embryo body stretches and its caudal end faces the blastopore. At this stage, neuroectoderm differentiation also occurs. In zebrafish, at the beginning of gastrulation, cells that maintained the germplasm segregate to form the primordial germ cells (PGCs). These cells are located at four random points on the embryo's axis on the edge of the blastoderm, more specifically on the hypoblast (Braat et al., 1999; Raz, 2003). The germplasm has a maternal origin and is evident in oogonia and spermatogonia, using transmission electron microscopy. It is formed by electron-dense structures known as nuages, which have mitochondria associated to ribonucleoproteins specific to the germ line (Santos et al., 2006). In embryos, PGCs can be recognized in routine histological sections due to their large size, slightly stained cytoplasm, and a nucleus with evident nucleolus (Braat et al., 1999). To identify the migration pathways of the PGCs during embryonic development, molecular markers present in the germplasm such as Vasa protein are used (Braat et al., 1999; Raz, 2003).

Blastopore closure

This event is important in embryonic development because only fertilized eggs complete this phase (Fig. 13.13I). Unfertilized eggs are divided by parthenogenesis and progressively degenerate during embryonic development, causing losses to fish farming. In general, fertilization rates are determined soon after the blastopore closure because unfertilized eggs degenerate before this stage.

Germ layer differentiation

The hypoblast forms the mesoderm, endoderm, and notochord while the epiblast forms the ectoderm and its derivatives (Fig. 13.13J). At this stage, the cranial and caudal ends of the embryo are evident.

Somitogenesis

The somites become evident, the heart starts beating, and the optic cup and optic vesicle are formed (Fig. 13.13K). In zebrafish, PGCs migrate until the first somite, laterally forming two groups. Progressively, the PGCs reach the eighth somite, where they find the primordial gonads (Braat et al., 1999; Raz, 2003).

Hatching

Successive contractions of the myotomes lead to the tail release (Fig. 13.13L). Vigorous muscular contractions of the embryo cause chorion rupture and hatching of the larva.

The duration of embryogenesis varies depending on egg size and the reproductive strategies of species (Nakatani et al., 2001; Sato et al., 2003a). Hence, fish with adhesive eggs and parental care behavior, such as the trahiras *Hoplias malabaricus* and *Hoplias lacerdae*, have a more prolonged embryo development (that is, 48 and 50 h, respectively at 24°C) than that of species with free eggs and do not care for the offspring such as the catfish *P. maculatus* and *P. corruscans* (18 and 19 h, respectively at 24°C). Egg adhesiveness, released egg diameter after induced spawning, and times at blastopore closure and hatching of some fish species are shown in Table 13.5.

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TABLE 13.5 Oocyte adhesiveness, diameter of the spawned oocyte (mean±DP) after induced spawning, and times at blastopore closure and hatching at 24°C during embryogenesis of fish species from the São Francisco River basin, Brazil.

Species/Authority	Oocyte adhesiveness	Spawned oocyte diameter (μM)	Blastopore closure (H)	Hatching (H)	References
Characiformes					
Astyanax bimaculatus (Linnaeus, 1758)	Weakly adhesive	1027.0±30.8	5 h	17 h	Sato et al. (2003a), Weber et al. (2013a, b)
Brycon orthotaenia (Günther, 1864)	Nonadhesive	1479.6±53.2	8 h	18 h 30 min	Honorato-Sampaio et al. (2015)
Curimatella lepidura (Eigenmann and Eigenmann, 1889)	Weakly adhesive	1019.5±20.6	7 h 30 min	25 h	Sato et al. (2003a), Perini et al. (2013)
Leporinus obtusidens (Valenciennes, 1837)	Nonadhesive	1245.9±39.2	7 h 50 min	19 h	Honorato-Sampaio et al. (2015)
Leporinus piau (Fowler, 1941)	Nonadhesive	1347.8±49.3	6 h 25 min	21 h	Sato et al. (2003a), Borçato et al. (2004)
Leporinus reinhardti (Lütken, 1875)	Nonadhesive	1067.0±55.1	6 h 30 min	17 h	Sato et al. (2003a)
Hopleritrinus unitaeniatus (Spix and Agassiz, 1829)	Adhesive	1103.1±132.9	18 h	44 h	Gomes et al. (2007)
Hoplias lacerdae (Miranda Ribeiro, 1908)	Adhesive	1522.1±230.4	21 h	50 h	Gomes et al. (2007)
Hoplias malabaricus (Bloch, 1794)	Adhesive	1222.8±134.3	19 h	48 h	Gomes et al. (2007)
Prochilodus argenteus (Spix and Agassiz, 1829)	Nonadhesive	1583.5±38.2	8 h 40 min	19 h 20 min	Honorato-Sampaio et al. (2015)
Prochilodus costatus (Valenciennes, 1850)	Nonadhesive	1511.6±46.6	7 h	16 h	Sato et al. (2003a), Rizzo et al. (2002)
Salminus franciscanus (Lima and Britski, 2007)	Nonadhesive	1613.2±59.5	7 h 30 min	18 h 50 min	Honorato-Sampaio et al. (2015)
Steindachnerina elegans (Steindachner, 1875)	Weakly adhesive	978.8±29.2	7 h 15 min	20 h	Sato et al. (2003a), Perini et al. (2013)
Siluriformes					
Franciscodoras marmoratus (Lütken, 1874)	Adhesive	1270.0±44.8	10 h 35 min	47 h	Sato et al. (2003a), Weber et al. (2012)
Pimelodus maculatus (Lacepède, 1803)	Nonadhesive	1113.9±37.0	7 h 30 min	18 h	Arantes et al. (2013)
Pseudoplatystoma corruscans (Spix and Agassiz, 1829)	Nonadhesive	1038.4±42.6	6 h	19 h	Sato et al. (2003a), Cardoso et al. (1995)
Rhinelps aspera (Spix and Agassiz, 1829	Adhesive	1360.8±40.9	9 h 40 min	45 h 50 min	Perini et al. (2010)
Rhamdia quelen (Quoy and Gaimard, 1824)	Nonadhesive	1471.7±47.6	8 h	25 h 30 min	Amorim et al. (2009)

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Chapter 14

The brain-pituitary-gonad axis and the gametogenesis

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Introduction

Like other vertebrates, fish reproduction is regulated by the interaction of the nervous and endocrine systems, and this interaction is carried out by the hypothalamus-pituitary-gonad or brain-pituitary-gonad (BPG) axis (Fig. 14.1). The organs that compose the axis produce and release, as internal factors, the hypothalamic, pituitary, and gonadal hormones. In addition, and interacting with the hormones produced by the axis, several internal factors such as neurotransmitters and neuromodulators and external factors such as temperature, photoperiod, and rainfall are involved.

In general, the hypothalamus synthesizes and liberates the gonadotropin-releasing hormone (GnRH), which, regulated by kisspeptins, stimulates the adenohypophyseal gonadotropic cells to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins stimulate steroidogenesis by the gonads through a complex pathway of specific enzymes. From now, we will address the histophysiology of each of the organs that integrates the BPG axis as well as aspects of their endocrine control.

Hypothalamus

Located in the ventral diencephalon, the hypothalamus is an important point of contact between the nervous and endocrine systems, connecting the brain to the pituitary gland through the pituitary peduncle. Such anatomical and functional connection is made differently in teleosts when compared with the other vertebrates, once teleosts do not have a pituitary-portal system.

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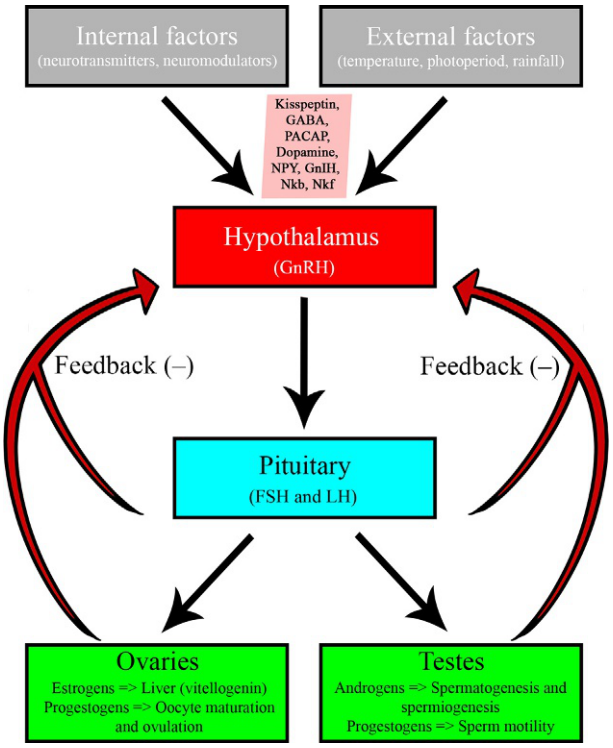

FIG. 14.1 Representative scheme of the BPG axis where the main organs that control the reproduction of teleosts are observed as well as the main hormones involved in this process. Kisspeptins (Kiss) are produced by hypothalamic neurons, and possibly can integrate external signs such as temperature, photoperiod, and rainfall with internal factors of the animal, such as other hormones, neurotransmitters, and neuromodulators. When stimulated, the gonadotropin-releasing hormone (GnRH) stimulates the release of the two gonadotropins, the follicle stimulating hormone (FSH) and the luteinizing hormone (LH) by pituitary gonadotropic cells. Gonadotropins act on the female and male gonads, stimulating the development of gametes and gonadal steroidogenesis. In turn, the gonadal steroids act on the gonads themselves and participate in the negative feedback of the process, controlling the upper part of the axis.

In teleosts, the specific groups of neurons in the hypothalamus (hypothalamic nuclei) that synthesize and secrete neuropeptides functionally linked to reproduction and other physiological activities directly innervate the pituitary gland through its nerve fibers, which bond together to form the neurohypophysis.

In this way, the branches of the neurohypophysis penetrate the adenohypophysis among the secretory cells, and their fibers end in the vicinity of these cells, releasing their neuropeptides. On the other hand, in mammals, birds, reptiles, amphibians, and other groups of fish, similar neuropeptides are released by nerve endings in the median eminence, located in the region of the pituitary peduncle. They enter the blood capillaries present there, which leads these molecules to the adenohypophysis by the pituitary-portal circulation (Gore, 2002; Peter, 1983, 1986).

There are many neurohormones and neuropeptides produced in the hypothalamic region that modulate the reproductive axis. The most studied is the gonadotropin-releasing hormone (GnRH) that, regulated by kisspeptins, is one of these peptides that participates in the first levels of the hormonal cascade derived from the BPG axis (Figs. 14.1 and 14.2). GnRH is synthesized during the pre- and postnatal life (Ebling, 2005; Trudeau, 1997), and its synthesis during embryonic development plays a crucial role in establishing the GnRH system that later in adult life will be activated to initiate and maintain puberty (Whitlock et al., 2006).

The first studied form of GnRH was from swine and sheep, now known as mammalian GnRH (mGnRH). In 1983, Sherwood and colleagues (Sherwood et al., 1983) determined the primary structure of chum salmon (*Oncorhynchus keta*) GnRH (sGnRH) pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH2, which, in turn, became considered the fish GnRH. From this discovery, the studies have been intensified, not only for the potential application in aquaculture but also for the possibility of studying this molecule from an evolutionary point of view from basal vertebrates such as fish (Gorbman and Sower, 2003; Lethimonier et al., 2004; Sherwood and Adams, 2005). To date, 30 GnRH variant forms are known in invertebrates and vertebrates, and nine of these distinct forms were isolated from the brains of fish species (Zhang et al., 2008).

Several isoforms of different fish species have been described, such as African catfish (*Clarias gariepinus*) (cfGnRH) and sea bream (*Sparus aurata*) (sbGnRH), among others. Following that current nomenclature rule, each new GnRH molecule sequenced receives the name of the species from which the GnRH was obtained, even though the same molecule could also be present in other species.

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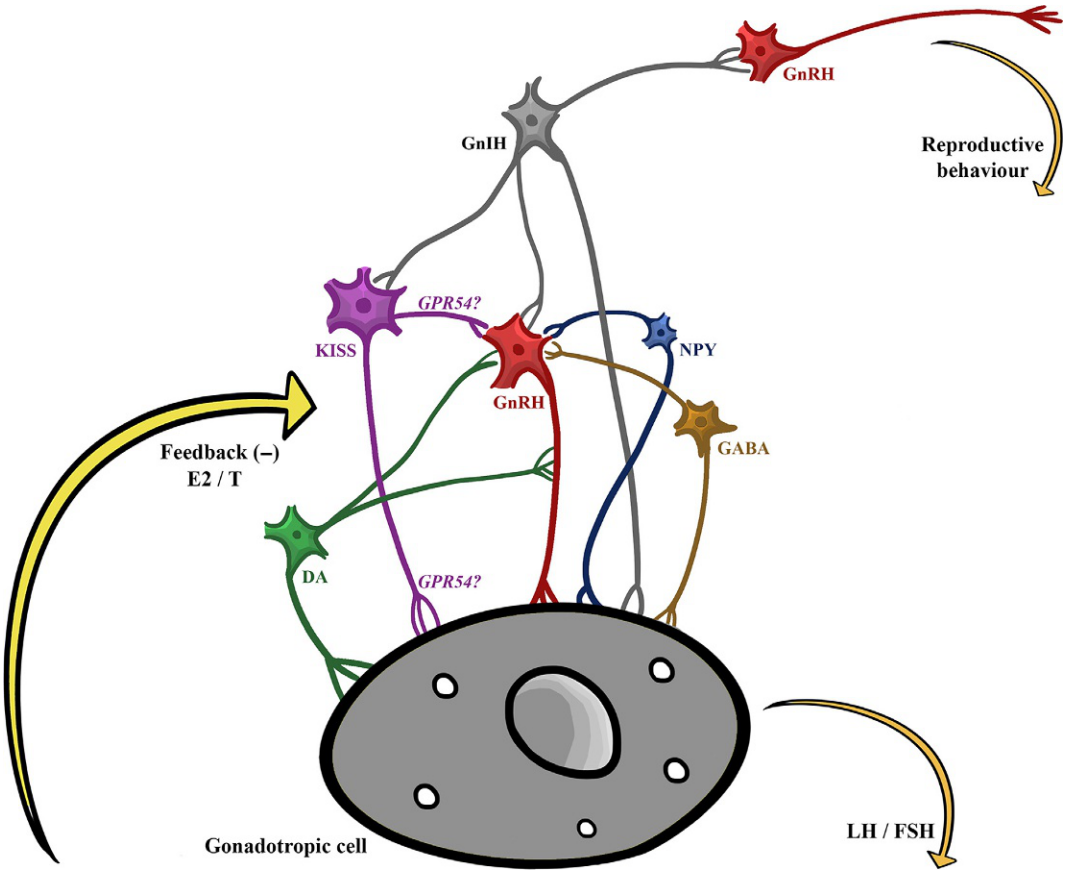

FIG. 14.2 Neuroendocrine pathway involving Fsh and Lh secretion in the pituitary gland. In most fish species, GnRH and dopamine directly stimulate and inhibit, respectively, the release of gonadotropins by the gonadotropic cells. These effects are modulated by GABA-expressing neurons that act to increase the GnRH secretion and inhibit dopamine release. Gonad steroids modulate the activity of this pathway directly through dopamine and GABA neurons, and, indirectly, possibly through the kisspeptin-producing neurons that innervate GnRH neurons. In addition, GnIH neurons can stimulate or inhibit directly or indirectly the gonadotropin secretion, depending on the fish species studied. In addition, more studies are needed to clarify whether kisspeptin neurons can integrate information such as the photoperiod and nutritional status of the animal. *(Drawing adapted from Zohar, Y., Muñoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol. 165(3), 438–55.)*

However, a new nomenclature for the variant forms of GnRH has been adopted for all vertebrates, based on the phylogenetic analysis of the known sequences and their respective sites of expression. In this sense, the existence of three main branches of GnRH has been demonstrated: the first one, containing GnRH-1, expresses hypophysiotropic variants, is species-specific, and is located mainly in the hypothalamus and preoptic area. The second branch contains GnRH-2, generally represented by the avian GnRH variant, cGnRH-II, which is expressed in the midbrain. The third type, GnRH-3, is group-specific, and is currently considered with only the salmon (or fish) GnRH isoform, expressed in the rostral telencephalon and olfactory bulb (Zohar et al., 2010).

Considering the mentioned isoforms, GnRH-1 is the one that acts directly on the pituitary, once, through receptors (GnRHRs) located mainly in PPD, stimulates the gonadotropic cells to synthesize and release gonadotropins, which stimulate the production of steroid hormones by the gonads. GnRH-2, present in the midbrain, as well as GnRH-3, located in the anterior portion of the brain and olfactory bulb, have a neuromodulatory function with implications for the modulation of sexual behavior (Soga et al., 2005). In the case of species that only have two types of GnRH, as in salmonids, GnRH-3 or sGnRH acts as GnRH-1 and -3, localizing in both the hypothalamus-preoptic area (such as GnRH-1) and in the telencephalon and olfactory bulb (such as GnRH-3).

The location of different types of GnRH in the nervous system and the colocalization of isoforms of this molecule were carried out using antibodies and ribosondes for GnRH-associated peptides (GAPs). This protein is coexpressed with its specific GnRH and has a relatively larger size. In addition, the primary GAP sequences were not conserved during evolution and, therefore, compared to the antibodies developed for GnRH, they are less likely to cross-react with the other isoforms (González-Martínez et al., 2001, 2002; Pandolfi et al., 2005).

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Studies related to GnRH in South American species are still scarce in the literature, but are representative of groups of economic and academic interest. There are studies related to the location and distribution of sGnRH, sbGnRH, and cGnRH-II in the brain of *Cichlasoma dimerus* (Pandolfi et al., 2002, 2005); in *Prochilodus lineatus* (Somoza et al., 1994); in *Odontesthes bonariensis* (Montaner et al., 2001; Somoza et al., 2002); and *Piaractus mesopotamicus* (Powell et al., 1997). In the latter, three molecular forms of GnRH present in the brain and pituitary gland were identified: sGnRH, cGnRH-II, and sbGnRH, and sbGnRH expression was higher in males than in females at the same stage of the reproductive cycle (Powell et al., 1997).

In *Astyanax altiparanae*, using the GF6 antibody that recognizes all forms of GnRH in teleosts, the pattern of distribution of cell bodies and immunoreactive fibers to GnRH has been described (Gomes et al., 2013). Next, employing molecular cloning, the complete cDNAs for preproGnRH-2 and -3 were isolated and characterized, demonstrating the absence of GnRH-1 in this characid species. Also, studies *in vivo* with the injection of the respective recombinant peptides in females able to spawn have demonstrated that in *A. altiparanae*, only GnRH-2 was able to stimulate the induced reproduction and culminate to spawning. Only GnRH-2 promoted the increase of *lhb* transcripts, in parallel to an increase of 17α-20β-DHP plasma levels (Chehade et al., unpublished data).

The distribution of GnRH forms in catfish *Steindachneridion parahybae* females was evaluated using heterologous antisera against GAPs (Honji et al., 2018). In this species, only two forms in the brain were detected, corresponding to catfish GnRH (cfGnRH or GnRH-1) and chicken-II GnRH (cGnRH-II or GnRH-2). Immunoreactive GnRH-1 fibers were detected in the pituitary gland, suggesting that this form may act as the hypophysiotropic one in this catfish (Honji et al., 2018).

Besides to the well-known role in regulating gonadotropic activity, Pandolfi et al. (2009), in a study with *C. dimerus*, observed that GnRH is involved in the control of somatolactin, a pituitary hormone exclusive to fish. In addition, this hormone participates in several physiological processes, including adaptation to the environment, and seems to have a role in reproductive processes (Cánepa et al., 2008).

It is currently postulated that each vertebrate species expresses two or three forms of GnRH in various tissues, and that GnRHs exert pleiotropic actions via several classes of receptors (Zohar et al., 2010). In mammals and in teleosts, some studies have detected the gene or protein expression of GnRH and its receptors in several extrahypothalamic and extrahypophyseal sites, such as, for example, in ovaries (Corchuelo et al., 2017; Park et al., 2013). Studies involving the analysis of hypothalamic and extrahypothalamic expression of GnRH as well as the molecular characterization and expression of the GnRH receptor (GnRHRs) in neotropical species are scarce.

In *O. bonariensis*, only two forms of GnRH (sGnRH and pjGnRH) were detected in the ovaries, differing from the three forms found in the brain. Furthermore, a correlation was observed between the sGnRH gene expression levels in relation to gonadal development, suggesting a function for this form in ovarian development (Guilgur et al., 2009). In addition, a preliminary study immunodetected GnRH in the ovaries of *A. altiparanae*, using the BB-8 antibody, which recognizes several forms of GnRH (unpublished data). Immunostaining was detected in the follicular and theca cells of oocytes from early to vitellogenic development, but also in the oocyte cytoplasm. These findings suggest that GnRHs present in somatic and germ cells are important for the development, survival, and selection of different groups of follicles that will develop asynchronically during the reproductive season in this species (unpublished data). However, further studies should be developed to identify the GnRH forms present in the *A. altiparanae* ovaries and their possible roles.

The complexity of neuroendocrine control of reproduction in teleosts is being increasingly understood. Recent studies involving genome editing techniques, such as transcription activator-like effector nuclease (TALEN) and the clustered regularly interspaced short palindromic repeats-*associated protein-9 nuclease* (CRISPR/Cas9), have shown surprising results on this intricate physiological regulation in fish. In zebrafish, *Danio rerio*, the knockout for GnRH-3 does not inhibit reproduction. Even though gonadotropin subunit expression was altered in young animals, the expression levels were normal in adults, suggesting the existence of compensatory mechanisms for the lack of GnRH-3 (Spicer et al., 2016). Moreover, even the double knockout animals for GnRH-3 and GnRH-2 were fertile. On the other hand, medaka GnRH-1 (hypophysiotropic form) knockout females did not alter the ovarian development but failed to ovulate, once *lhb* transcripts are downregulated in these animals. However, GnRH-1 knockout males remain fertile (Takahashi et al., 2016). All these results lead us to question whether the GnRH system is essential for the reproduction of the teleost and even if the resulting phenotypes are gender or species-specific.

GnRH secretion is controlled by several environmental factors such as photoperiod, temperature, rainfall, pheromones, and amount of food available. In addition to these factors, there are many other neuropeptides that also participate in the control of the reproductive axis through GnRH stimulation or inhibition (Figs. 14.1 and 14.2). Among these is dopamine, a neurotransmitter with a small molecular weight that in most teleosts inhibits the secretion of gonadotropins directly through receptors in pituitary cells (Chang and Peter, 1983), or indirectly through receptors in the nerve endings of GnRH of the neurohypophysis, or in the GnRH-secreting neurons themselves, inhibiting their release (Yu et al., 1991). In addition, dopaminergic inhibition has different physiological and adaptive meanings depending on the species of teleost analyzed. In

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goldfish, dopamine prevents ovulation when environmental conditions are not appropriate, and in males of the same species, dopamine prevents spermiation prior to ovulation of the female partner (Sloley et al., 1992). For this reason, a combination of treatments with GnRH agonists and dopamine antagonists, such as pimozide, is extremely effective for the induction of ovulation, and consequently spermiation in this species (Peng et al., 1994).

The gamma amino butyric acid (GABA) is a neurotransmitter acting on the HPG axis, being extremely abundant in the brain of all vertebrates where it acts to stimulate the secretion of LH and GnRH (Kah et al., 1992; Sloley et al., 1992) and inhibit the secretion of dopamine (Trudeau, 1997; Trudeau et al., 2000).

The neuropeptide Y (NPY) is related to feeding regulation in fish and mammals, besides stimulating the synthesis of growth hormones (Peng et al., 1993c) and participating in the regulation of gonadotropin secretion (Breton et al., 1991; Peng et al., 1993b), either in the pituitary or through GnRH (Peng et al., 1993a; Trudeau, 1997). For these reasons, it can be considered a mediator for the growth, nutritional status, and reproduction in these species.

Identified in birds for the first time, and subsequently in mammals and other vertebrates, the gonadotropin-inhibitory hormone (GnIH) is a dodecapeptide member of the RFamide peptide family, which in turn includes kisspeptins and neuropeptide FF, among others. The first studies demonstrated that GnIH acts directly on the pituitary gland, inhibiting the synthesis and release of gonadotropins (Tsutsui et al., 2007; Tsutsui and Osugi, 2009; Tsutsui and Ukena, 2006; Yin et al., 2005) besides exerting neuromodulatory function in the GnRH-producing neurons (Bentley et al., 2006, 2008). GnIH synthesis is modulated by melatonin, which suggests its role in translating photoperiod signals to other endocrine centers involved in reproductive control (Tsutsui et al., 2007). However, further studies are needed to clarify the functions of this neuropeptide in fish and especially in neotropical fish species. The relationship of GnIH with the inhibition of reproduction in fish is still little studied, and for this reason, many authors have preferred to name it the LPXRFamide peptide (Muñoz-Cueto et al., 2017).

The anatomical relationships between GnIH and GnRH-producing neurons are well known in birds and mammals, as well as the occurrence of GnIH fibers toward the median eminence. In fish, however, not all species seem to have direct innervation in the pituitary gland by GnIH-fibers while the anatomical relationships between the neurons that synthesize these two neuropeptides seem to vary from species to species (Muñoz-Cueto et al., 2017).

The anatomical distribution of the GnIH-producing neurons in the brain of *C. dimerus* was investigated, showing that these neurons are in the nucleus *posterioris periventricularis* and in the nucleus *olfacto-retinalis*. On the other hand, GnIH-immunoreactive fibers were widely distributed throughout the brain, especially in the nucleus *lateralis tuberis*, but not innervating the pituitary gland (Di Yorio et al., 2016). Also, once GnIH was able to inhibit gonadotropin release in this species (Di Yorio et al., 2016), the authors also investigated the relationships between GnIH and GnHR forms-producing neurons. No apparent contact between GnRH-1 and GnIH neurons was detected, but only fiber-to-fiber interactions between GnRH-2 and GnIH. Also, colocalization of GnIH and GnRH-3 in neurons of the *nucleus olfacto-retinalis* was detected, demonstrating an interaction of these neuropeptides that controls reproduction in this fish species (Di Yorio et al., 2018).

Another important neuropeptide is kisspeptin (Kiss), first identified in mammals (De Roux et al., 2003; Seminara et al., 2003), which is known to play a role in the neuroendocrine regulation of reproduction. Kisspeptine 10 (peptide with 10 amino acids), one of the peptides derived from the cleavage of the molecule, is highly conserved among vertebrates.

Kanda et al. (2008) were pioneers in the cloning of the Kiss 1 gene sequence in teleosts, with medaka being the object of study. At the same time, the Kiss 1 gene was cloned into several species of teleosts such as zebrafish (Biran et al., 2008), zebrafish and pufferfish (Van Aerle et al., 2008), and zebrafish and medaka (Kitahashi et al., 2009), and the sequences from all these animals have been shown to be highly conserved. These results support the idea that Kisspeptine 1 plays an important role in various aspects of the neuroendocrine control of reproductive functions. The Kiss 2 gene, like Kiss 1, was also identified, especially in teleosts, where it probably shows functional activity. It is believed that this gene was lost during vertebrate evolution. Also, each ligand has its cognate receptor, Kiss 1R and Kiss 2R.

The Kiss 1/GPR54 system (a hormone and its receptor) is considered a trigger for GnRH, allowing integration between environmental and metabolic signals with the reproductive axis (Tena-Sempere, 2006). In fish, this issue is not yet conclusive, but it is known that Kiss 1R receptor expression occurs in GnRH neurons (Parhar et al., 2004) and is increased at the onset of puberty when compared to the pre- or postpubertal period while GnRH expression is also increased (Mohamed et al., 2007; Nocillado et al., 2007), indicating that GnRH cells are direct targets of kisspeptins in these animals. Furthermore, it is also suggested that kisspeptins induce GnRH release in puberty via interaction with Kiss 1 in fish, as occurs in mammals (Parhar et al., 2004).

In teleosts, the expression of kisspeptins occurs in the neuronal cell bodies of two encephalic nuclei, in the nucleus *posterioris periventricularis,* and in the nucleus *ventral tuberalis*. The Kiss 1 neurons of the nucleus *ventral tuberalis* display sexual dimorphism, being more numerous in males than in females. Interestingly, there is no dimorphism in the neurons of the nucleus *posterior periventricularis*. The two populations of Kiss 1 respond differently to steroid hormones, and only Kiss 1 cells in the nucleus *ventralis tuberalis* are upregulated by estrogens, suggesting the presence of

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positive feedback from the BPG axis during ovulation (Kanda et al., 2008). Kitahashi et al. (2009) also reported another neuronal group of Kiss 1 in the zebrafish and medaka habenula that may be involved in reproductive control.

In neotropical fish species, up to now, only Kiss 1, Kiss 2, Kiss 2R, and Kiss 3R were isolated and characterized in *O. bonariensis* (Tovar Bohórquez et al., 2017a). All these elements are widely distributed in adults, even in several non-reproductive tissues. Also, ontogenetic studies demonstrated a higher expression of the Kiss 2 gene, being related to differentiation for the HPG axis in males (Tovar Bohórquez et al., 2017b). The expression of these four genes was investigated in the brain, pituitary gland, ovaries, and testes in adult *O. bonariensis* in different stages of the reproductive cycle. Surprisingly, all transcripts were detected in all organs, and showed a specific expression profile related to the gender and gonadal cycle stage (Tovar Bohórquez et al., 2017a).

Finally, the scheme shown in Fig. 14.2, adapted from Zohar et al. (2010), summarizes the neuroendocrine control of gonadotropin secretion, considering the already consolidated knowledge and the data that still need to be elucidated.

Pituitary gland

The hypophysis or pituitary gland is a small endocrine gland located at the base of the diencephalon, being attached to it by a thin pituitary peduncle. In its ventral portion, it is accommodated in a depression of the sphenoid bone called the *sela turcica*. The pituitary has two parts: the adenohypophysis, consisting of different secreting hormones cells, and the neurohypophysis, consisting mainly of amyelinic nerve fibers originated from neurosecretory neurons located in the brain.

The teleost pituitary gland has some peculiarities such as the absence of a pituitary-portal system, as found in the great majority of vertebrates to control hormonal secretion by the central nervous system via portal blood circulation. Instead, in teleosts, the nerve fibers from the hypothalamic nuclei reach the adenohypophyseal cells, establishing contact or ending in the extracellular matrix next to them. These fibers carry the releasing or inhibitory factors of the secretion of adenohypophyseal hormones, such as the GnRH, for example. Another peculiarity is the distribution in groups of the different pituitary hormones producing cells in well-delimited regions of the adenohypophysis, as will be seen below.

Adenohypophysis

The adenohypophysis in teleosts is divided into two parts: pars distalis (PD) and pars intermedia (PI). PD, in turn, is subdivided into rostral pars distalis (RPD) and proximal pars distalis (PPD). The basis of this division is the arrangement of specific groups of secretory cells, as shown in Fig. 14.3. In this way, the RPD of the teleost pituitary contains prolactin (PRL) and corticotropin (ACTH) secreting cells, and, in some species, thyrotropin (TSH) secreting cells. PPD contains growth hormone (GH), gonadotropin (GtHs), and thyrotropin (TSH) secretory cells. In the PI, the secretory cells are melanotropin (MSH) and somatolactin (SL) producing cells, the latter a hormone exclusively found in fish.

Although secretory cells normally maintain the same pattern of distribution, being found in the same adenohypophyseal regions, different species of teleosts may have some variations, mainly depending on the stage of the reproductive cycle in which the animal is found. For example, it is common to find gonadotropin secretory cells at the periphery of PI in advanced maturation and mature stages.

The identification of adenohypophyseal cell types began to gain prominence in the literature with the study of Olivereau and Ball (1964). Studies were carried out by analyzing histological sections of the gland under the light microscope by conventional staining methods by hematoxylin and eosin (HE) and trichrome as Mallory and Mason, and more specific histochemical methods such as periodic acid-Schiff's reagent (PAS), Alcian Blue (AB), and hematoxylin-lead (HPb), methods widely used until today for recognition of the histological structure of the pituitary gland. Subsequently, immunohistochemical methods of hormone-specific labeling were performed (Olivereau and Nagahama, 1983; Schreibman and Margolis-Kazan, 1979). Excellent reviews are found on these issues in Ball and Baker (1969), Kawauchi and Sower (2006), Schreibman (1986), Van Oordt and Peute (1983), and Quesada et al. (1988).

Considering the great variety of neotropical freshwater teleosts, relatively few species have been studied in relation to the identification of pituitary hormone-producing cells. As examples of works with neotropical species, we have *Rhamdia hilarii* (Val-Sella and Fava-De-Moraes, 1979); *R. hilarii*, *Hypostomus punctatus*, and *Prochilodus scrofa* (Val-Sella et al., 1988); *P. mesopotamicus* (Borella et al., 1997); *Arapaima gigas* (Borella et al., 2009); *Salminus hilarii* (Honji et al., 2013b); and *Serrasalmus spilopleura* and *Pimelodus maculatus* (Nóbrega et al., 2017). The ontogeny of the pituitary gland in South American species was studied, for example, in *C. dimerus* (Pandolfi et al., 2001) and *Salminus brasiliensis* (Jesus et al., 2014).

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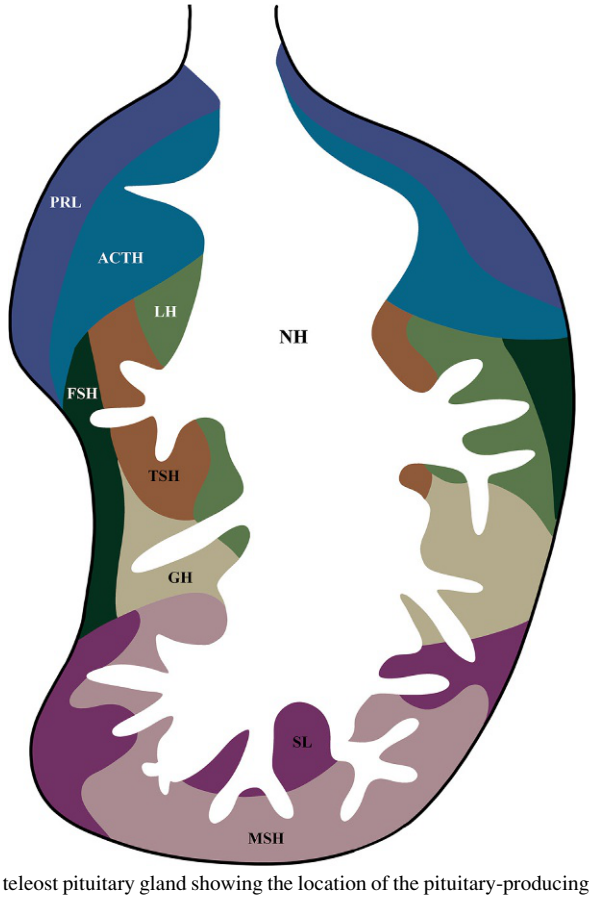

FIG. 14.3 Representative scheme of the teleost pituitary gland showing the location of the pituitary-producing hormone cells. Prolactin (PRL) and adrenocorticotropic hormone (ACTH) producing cells are found in the rostral pars distalis. Growth hormone (GH) and thyrotropin hormone (TSH) producing cells, as well as the gonadotropes—follicle stimulating (FSH) and luteinizing (LH) hormone producing cells—are found in the proximal pars distalis. The somatolactin (SL) and melanotropic hormone producing cells (MSH) are in the pars intermedia. Pars nervosa is represented by branches of the neurohypophysis (NH) that penetrate the gland by the rostral pars distalis, and branches through the proximal pars distalis in smaller and smaller portions until reaching the pars intermedia.

Currently, the production of highly specific antibodies and/or probes has made the recognition of glandular cells more accurate, as has occurred with gonadotropic cells.

Gonadotropic cells or gonadotropes are generally described as globular, cyanophilic, or basophilic cells with peripheral round nuclei that display vacuoles in the cytoplasm. They are also recognized by their changes in number and size, and in the number of vacuoles, throughout the reproductive cycle.

Concerning the presence of one or two gonadotropic cell types, the use of homologue antibodies also allowed the identification of two gonadotropes in most of the studied teleosts. A study in transgenic zebrafish demonstrated not only two different populations and arrangements of the gonadotropes, but also suggested different neural regulation of FSH and LH cells, made by neuroglandular regulation (as common for teleosts), and one unexpected neurovascular regulation, as present in tetrapods (Golan et al., 2015).

Gonadotropins (GtHs), as already mentioned, are the main hormones that modulate the reproductive function. They are currently called follicle-stimulating hormones (FSH) and luteinizing hormones (LH), by molecular and functional similarity with the gonadotropins of other vertebrates. This replaces the terms GtH I, or vitellogenic, and GtH II, or maturational, respectively, that were previously used. They are glycoprotein hormones whose molecules consist of two subunits, α and β. The α subunit is common to both gonadotropins and to thyrotropin (TSH) as well, and the β subunit is variable, conferring the specificity to the different hormones.

The cloning of the cDNA corresponding to these subunits allowed the localization of gonadotropin-producing cells by in situ hybridization or by homologous antibodies, confirming that, in general, FSH and LH are synthesized by different cell

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types in teleosts, not by a single cell type as in tetrapods. The popularization of molecular cloning techniques has allowed the isolation of genes coding for GtH subunits in several fish species, which until the one of the last reviewed data represented at least 14 orders of teleosts (Levavi-Sivan et al., 2010).

Concerning the neotropical species, the isolation of partial gonadotropin β subunit cDNAs was obtained for some Characiform species such as *S. hillari* (Moreira et al., 2015) and *P. mesopotamicus* (Kuradomi et al., 2016). Afterward, the total β subunits of both gonadotropins were obtained from pejerrey (Shinoda et al., 2010), *A. gigas* (Sevilhano et al., 2017), and *A. altiparanae* (Jesus et al., 2017). These are important reports, as antibodies against gonadotropin subunits from neotropical fish were not available until these reports. The identification of the cellular expression of gonadotropins in the pituitary was investigated before only in a few Characiformes species using heterologous antibodies, such as the studies on *A. gigas* (Borella et al., 1997), *S. hilarii* (Honji et al., 2013a,b), and *S. brasiliensis* (Jesus et al., 2014).

The two gonadotropins have different patterns of expression in the pituitary at different stages of the reproductive cycle in females of Salmoniformes (Nozaki et al., 1990), which display synchronous or group synchronous ovarian development. In this group, FSH acts in the early phase of the reproductive cycle, during vitellogenesis, and LH in the late phase, during final oocyte maturation and ovulation (Breton et al., 1998; Gomez et al., 1999). However, an increase of transcript levels for both gonadotropins was detected during the late phases in asynchronous ovarian development groups such as Cypriniformes, Perciformes, and Pleuronectiformes (Gen et al., 2000; Hassin et al., 2000; Kajimura et al., 2001; Sohn et al., 1999). Concerning neotropical species, which present a range of reproductive strategies such as synchronous oocyte development in two groups and total spawning or asynchronous oocyte development with multiple spawning, different roles for gonadotropins among different reproductive strategies are expected.

In adult males, FSH and LH stimulate spermatogenesis and testicular steroidogenesis. The androgens testosterone and 11-keto-testosterone (11KT) are produced by the Leydig cells, present in the interstitial tissues of the testes. In addition to the production of androgens, the gonadotropins stimulate the secretion of androgens and the secretion of progestogens, especially 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP), which is produced in greater quantity by the action of LH (Viziano et al., 1996; Weltzien et al., 2004).

As previously mentioned in this chapter, and showed in Figs. 14.4 and 14.5, in general, it is accepted for most fish species that FSH regulates the early stages of development and gametogenesis, stimulating the production of 17β-estradiol (E2) by the ovarian follicles, coinciding with vitellogenesis. In the testes, FSH reaches receptors in both the Sertoli and Leydig cells, acting in the early stages of spermatogenesis. Concerning LH, studies indicate that it acts on the advanced maturation of oocytes, being more active in stimulating the release of 17α,20β DP by postvitellogenic oocytes, which promotes ovulation. In males, LH also acts in the late stages of spermatogenesis and stimulates the production of androgens. In addition, studies performed in fish with semicystic spermatogenesis, where spermatids are free in the seminiferous lobule lumen and develop into spermatozoa without direct contact with the supporting Sertoli cells, show that haploid spermatids also express LH receptors, and LH is the effective hormone that promotes their differentiation into spermatozoa in vitro (Chauvigné et al., 2014, 2016).

The secretion of gonadotropins is under GnRH and other neurohormone regulation, as already seen in this chapter, but GtHs are also regulated by the feedback of gonadal steroids that completes the BPG axis.

Neurohypophysis

The neurohypophysis (NH) in teleosts consists of unmyelinated nerve fibers from neurosecretory neurons located in brain areas, especially the hypothalamic ones. Basically, NH encompasses the proximal portion of the pituitary peduncle and the nerve fibers that constitute the pars nervosa (PN), which are distributed throughout the AH and branch more intensely in the PI (Fig. 14.3). Parts of these fibers are originated from vasotocin (VT), isotocin (IT), and melanotropin concentrating hormones (MCH) producing neurons. On the other hand, in general, there is a correspondence between the distribution of the nerve fibers involved in the control of a certain cell type and the distribution of this cell type in the pituitary. For example, gonadotropin-releasing hormone (GnRH) immunoreactive fibers are preferably located in the PPD, where the gonadotropin-producing cells are located. Likewise, several neuropeptides and neurotransmitters that modulate gonadotropin secretion in vivo and in vitro have been identified in fibers that penetrate the PD, such as GnRH itself, gamma amino butyric acid (GABA), neuropeptide Y, dopamine, pituitary adenylate cyclase-activating peptide (PACAP), galanin, somatostatin, cholecystokinin, substance P, or the growth hormone-releasing factor (for reference, see Zohar et al., 2010) (Figs. 14.1 and 14.2).

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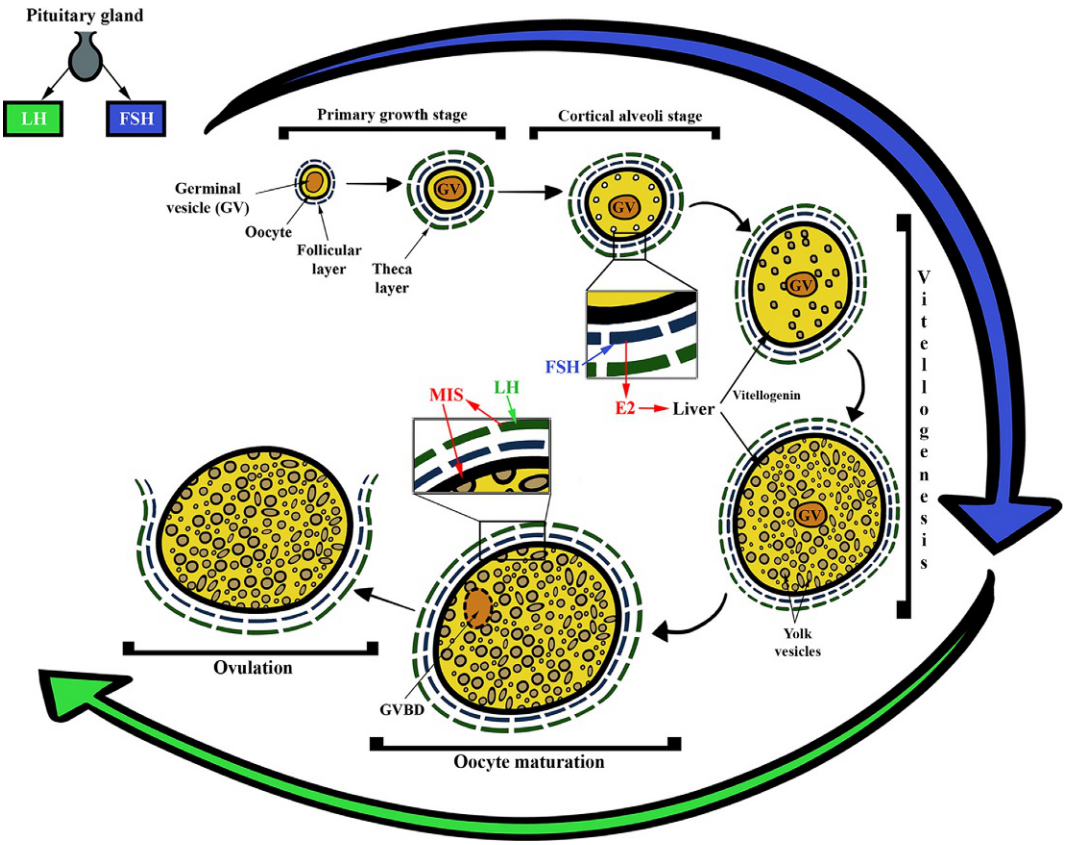

FIG. 14.4 Stages of oocyte development and hormonal control. Gonadotropins (FSH and LH) produced by the pituitary gland target the gonads and promote the development of the oocyte follicles from primary growth until maturation. Throughout this process, the oocyte is surrounded by two other cell layers, the follicular layer and the theca layer. These somatic cells are responsible for promoting the oocyte growth and survival through the provision of growth factors, hormones, and nutrients, among others. In addition, these cells are the targets of gonadotropins, so FSH acts on follicular cells stimulating the production of E2. This hormone targets the liver, promoting the production of vitellogenin, which in turn stimulates the progression of vitellogenesis and the consequent accumulation of yolk in the oocyte follicles. LH acts on the theca cells, promoting the production of MIS (or MIH) that targets the oocyte, stimulating the resumption of meiosis and germinal vesicle breakdown, and resulting in the ovulation process.

Oogenesis

As in all other vertebrates, the process of oocyte formation is widely conserved and, in general, is applied to all species of fish studied. Therefore, the vertebrate oocytes, which grow inside the ovarian follicles, stop in the first meiotic prophase. In teleosts, as in other nonmammalian vertebrates, the main events responsible for the enormous growth of oocytes are essentially due to the accumulation of yolk proteins in their cytoplasm (Devlin and Nagahama, 2002). After the oocyte completes its growth, it becomes ready for the next phase of oogenesis, that is, the resumption of meiosis (completion of the first meiotic division followed by progression to metaphase II), which is accompanied by several maturation processes in the nucleus and in the oocyte cytoplasm. This process, called oocyte maturation, occurs during ovulation and consists of germinal vesicle breakdown (GVBD), chromosomal condensation, meiotic spindle assembly, and formation of the first polar body (Nagahama and Yamashita, 2008).

In neotropical fish, with some variations of nomenclature, the following phases of oocyte development are found (Brown-Peterson et al., 2011; Quagio-Grassiotto et al., 2011): oogonia; the previtellogenic phases of single nucleolus, multiple nucleoli, and perinucleolar; the vitellogenic phases of cortical alveoli, initial vitellogenesis, advanced vitellogenesis, and complete vitellogenesis; and culminating with mature oocytes during the ovulation process. Regarding the ovarian maturation process (Brown-Peterson et al., 2011; Lowerre-Barbieri et al., 2011), descriptions obtained for captive and in nature animals are similar, presenting four common phases (with several subdivisions): "developing," "spawning capable," "regressing" or "spent," and "regenerating." In animals collected in the wild, the "regressing" or "spent" phase, which occurs after spawning, is naturally observed. In captivity, however, most rheophilic species are not able to release

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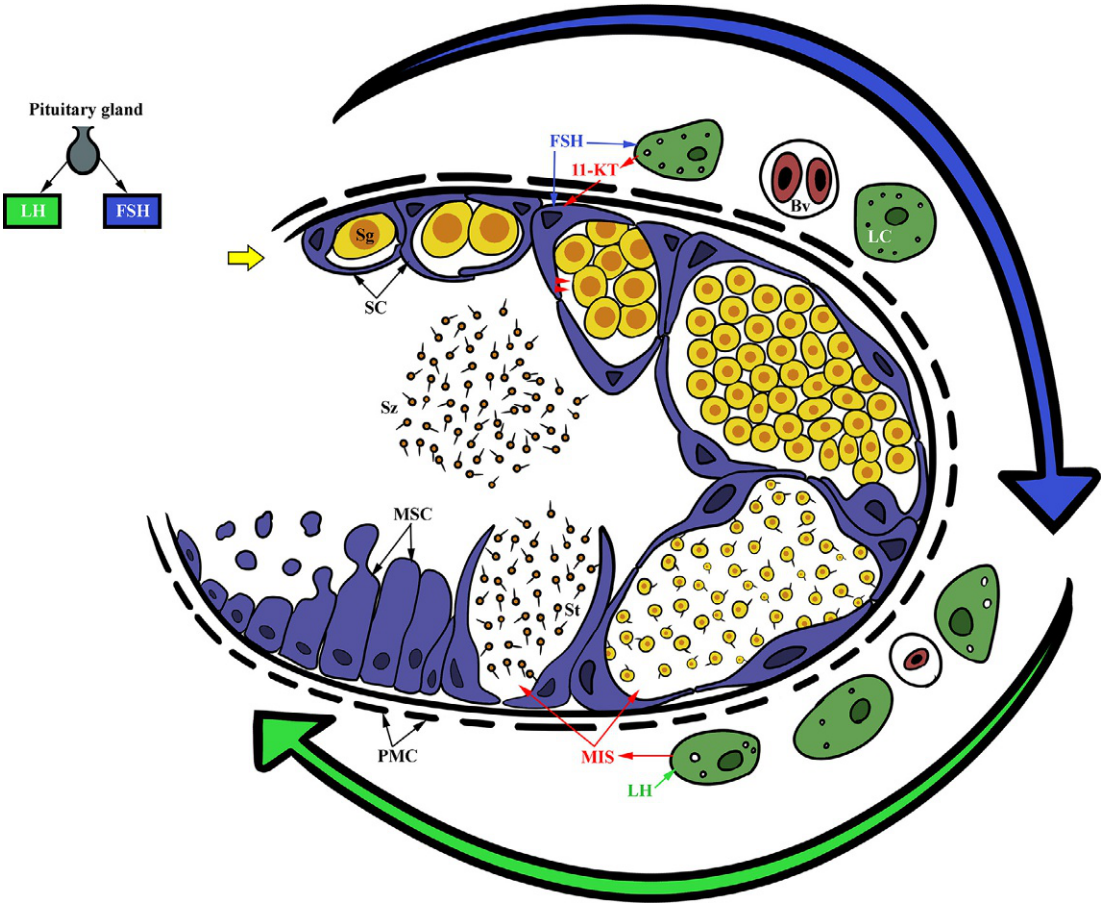

FIG. 14.5 Spermatogenesis and its hormonal control. Gonadotropins (FSH and LH) produced by the pituitary gland target the gonads and promote the development of the spermatic cell lineage from an undifferentiated cell, the spermatogonium (Sg), to a very specialized one, the spermatozoa (Sz). Throughout this process, the spermatic cells are surrounded by the Sertoli cells (SC) within the seminiferous tubules, which are limited by the peritubular myoid cells (PMC). In addition, some interstitial cells also display an important role in the maturation of the spermatic cells, like the Leydig cells (LC). These somatic cells are responsible for promoting the growth, survival, and differentiation of the spermatic cell lineage through the provision of growth factors, hormones, and nutrients, among others. In addition, these cells are the targets of gonadotropins, so FSH acts on Sertoli and Leydig cells, stimulating the production of 11-KT (*arrowheads*). This hormone stimulates the differentiation of the spermatogonia and the progression of the spermatogenesis. LH acts only on the Leydig cells, promoting the production of MIS (or MIH) that targets the spermatids (St) ,stimulating spermiogenesis, spermatozoa production, and the spermiation process.

their gametes and, if not hormonally induced, enter into a process of oocyte degeneration and gamete reabsorption. This is known as the "regenerating" phase, without going through the previous phase.

Dynamics of oocyte development and spawning types in freshwater fish used in fish farming

In relation to the native species of commercial importance, most of them are migratory and present dynamics of (1) "synchronous oocyte development in two groups" and total spawning, or (2) "asynchronous oocyte development" with multiple spawning. In general, ovaries from the first group display two batches of oocytes, one from mature oocytes for spawning and the other from previtellogenic oocytes, which must be recruited for the next breeding season. In the second group, ovaries have oocytes at all stages of development and their elimination occurs as they reach full maturation.

In rheophilic species, the pattern of oocyte development and the respective reproductive cycles have been described in fish of several groups, mainly Characiformes. In the latter group, the so-called "round fish" have been studied such as the tambaqui, *Colossoma macropomum* (Vieira et al., 1999), and the pacu, *P. mesopotamicus* (Lima et al., 1991; Romagosa et al., 1993a; Schorer et al., 2016). Still in this order, fish from the genus *Brycon*, known for their importance in sport fishing, have also been studied as the matrinxã, *Brycon amazonicus* (Romagosa et al., 2002), and the piracanjuba, *Brycon*

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orbignyanus (Ganeco et al., 2001). In addition, we further highlight the large Siluriform catfish represented by several families, including species of the genus *Pseudoplatystoma*, such as the pintado, *Pseudoplatystoma corruscans* (Crepaldi et al., 2006), and the cachara, *Pseudoplatystoma fasciatum* (Romagosa et al., 2003). We also highlight studies on the reproductive cycles of several Amazonian species in natural environments, such as the mapará (*Hypophthalmus edentatus*) (Carvalho, 1980) and the duck catfish (*Ageneiosus ucayalensis*) (Oliveira et al., 2017).

In another aspect, it should be clarified that not all species of importance for fish farming carry out reproductive migrations, with some species spawning locally and often presenting breeding periods not as rigid as migratory species. In this group, there may be a tendency to intensify breeding and spawning in the warmer summer months, but individuals may breed throughout the year. Because they spawn several times, these species often have an oocyte developmental mechanism of the asynchronous type. As previously mentioned, there are several batches of oocytes in different stages of development, guaranteeing the presence of mature oocytes at different times throughout the year (more details on this type of spawning can be found in Lubzens et al., 2010).

Among the numerous native species that present an asynchronous oocyte development mechanism, we can mention some species of fish farming and aquaculture importance such as the jundiá, *Rhamdia quelen* (Ghiraldelli et al., 2007; Reidel et al., 2010) and *Rhamdia voulezi* (Bittencourt et al., 2018), the piranha, *Serrasalmus brandtii* (Honorato-Sampaio et al., 2009), and the pirarucu, *A. gigas* (Godinho et al., 2005; Chu-Koo et al., 2009; Torati et al., 2016). In this group, pirarucu is one of the largest species of freshwater fish in the world, very appreciated for the quality of its meat and high carcass yield, which provide great blankets of meat devoid of thorns. Still regarding fish species with asynchronous oocyte development, and despite being migratory species, we have the lambaris (Characiformes), mainly the lambari-do-rabo-amarelo—*A. altiparanae* (Chehade et al., 2015; Cassel et al., 2017)—which is used as bait in sport fishing, ration, and for direct consumption. The lambari deserves special mention also for being used as a fish model species for groups that study the reproduction of native species (Gomes et al., 2013; Adolfi et al., 2015; Camargo et al., 2017; Tolussi et al., 2018; among others) and for being a native species whose zootechnical potential is being exploited by means of techniques that involve the production of triploid animals.

Oocyte development in neotropical migratory fish

In recent decades, numerous studies have described the reproductive cycle of neotropical species, both in the natural environment and in captivity. Among the various studied species, most of them show a similar, logical, and sequential pattern throughout the oocyte development process, which reflects and determines the so-called "stages of ovarian development." Studies developed with species collected in natural environments describe, with some variations, five stages of development: immature (animals that have never reproduced and that are often not found in collections), developing (in which the ovaries begin to develop, but are not yet ready to spawn), spawning capable (usually subdivided into phases, the specimens display complete ovarian development and are physiologically capable of reproducing), regressing (end of reproduction, postspawning), and regenerating (sexually mature but reproductively inactive specimens) (Zaniboni-Filho and Resende, 1988; Menezes et al., 2000; Veregue and Orsi, 2003; Alvarenga et al., 2006; Brown-Peterson et al., 2011; and several others). Similarly, in captive animals, five phases are observed, and there may be some variations (Lima et al., 1991; Romagosa et al., 1993a; Ganeco et al., 2001; Chehade et al., 2015; Schorer et al., 2016; Cassel et al., 2017; among others).

Early oocyte development

The early oocyte development (primary growth) (Fig. 14.4) usually occurs in autumn and early winter, between May and June in regions with a tropical climate (Hainfellner et al., 2012a). However, in regions with an equatorial climate (warmer), this period may be different or occur concurrently with the presence of individuals of the same species in other, more advanced stages of development (Vieira et al., 1999). In this period, there is an intense synthesis and accumulation of cellular substances and organelles in the oocytes that will be responsible for part of the yolk production (the nutritional reserve of the egg, responsible for the survival of the embryo) (detailed reviews of this process in Guraya, 1986; Nagahama and Yamashita, 2008; Lubzens et al., 2010). At this stage, tiny ovaries are often referred to as fillets difficult to see, especially in smaller species. In general, ovarian cells are found in four distinct developmental stages: oogonia, primary oocytes in the "single nucleolus" stage, primary oocytes with "multiple nucleoli," and primary "perinucleolar" oocytes (Narahara et al., 1988; Romagosa et al., 2002; Cassel et al., 2017; among others).

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Oogonia

Oogonia are tiny diploid germ cells that pass through mitotic divisions and originate the primary oocytes. In general, these cells have a large nucleus and a small amount of cytoplasm (Guraya, 1986). In this context, the characteristics of the oogonia proliferation process (mitotic division) as well as the genetic, hormonal, and biochemical factors involved in its transformation into primary oocytes are practically unexplored themes in native species. However, we must consider that this is a complex process that has not yet been fully elucidated in any vertebrate species (more details in Lubzens et al., 2010). Indeed, given their morphological similarity to newly formed primary oocytes, oogonia are often confused with these cells, both of which have a similar size and morphology and occupy the same regions of the ovary, the so-called "oogonia nests." However, in electron microscopy, the oogonia are more electron-lucid than the other germ cells, and their cytoplasm is full of mitochondria, several nuages near the nuclear membrane, prominent Golgi apparatus, and *lamella annulata* (Cassel et al., 2017).

In addition, the kinetics of the oogonia division are a matter of extreme importance, not only of a basic nature but also with direct implications for reproduction and application in the fish farming of captive breeding species. The number of mitotic divisions that an oogonium suffers before generating the primary oocyte is ultimately the main factor determining the fecundity of matrices. That is, the number of oocytes released by a female, in absolute numbers or in relation to body or ovary weight, is related to the proliferation of oogonia before they enter meiosis. In this context, it is known that the last mitotic division of the oogonium generates only a pair of primary oocytes, and each primary oocyte, in turn, generates a single secondary oocyte (and a polar corpuscle), which is eliminated by the female (together with the second polar corpuscle) in the reproductive act (Guraya, 1986; Lubzens et al., 2010). Thus, with a simple mathematical analysis, it is evident that the higher the proliferation rate of oogonia, the greater will be the number of primary and secondary oocytes, and consequently, the greater will be the fecundity of the species.

It must be clear that the proliferation rate of oogonia is not the only factor that interferes in the fecundity of the species, and other factors such as the mechanisms of apoptosis, oocyte atresia, and various interferences at the time of induced reproduction are phenomena that also need to be considered and better studied. Differently from females, in males this subject has been intensively studied. It is known that each species has a fixed number of spermatogonia divisions, and that this type of mitotic division, together with the apoptotic mechanisms, is a determinant in the sperm production capacity of species (for review see Nóbrega et al., 2009; Schulz et al., 2010).

Lubzens et al. (2010) reviewed the molecular and hormonal mechanisms that control the proliferation of oogonia. This subject remains unknown in native species, but it is known that, in general, in ovaries still "developing," the oogonia divide mitotically, producing cell groupings called "oogonia nests." These newly formed groups are heterogeneous and show, in addition to oogonia, newly formed oocytes and prefollicular cells. Thus, in these cell groups, oogonia and primary oocytes appear side by side. In this context, a detailed description of this initial stage of oogenesis, including the relationship of germ cells to the ovarian germinal epithelium, was presented by Grier et al. (2007) on rainbow trout ovaries. Due to the high degree of conservation of the initial process of oocyte formation between species (Lubzens et al., 2010), it is very likely that this process in rheophilic fish will be similar to those described for other species of fish, a detail briefly showed for *A. altiparanae* by Cassel et al. (2017).

Primary oocytes in the "single nucleolus" stage

After the pachytene step of the first meiotic division, when the homologous chromosomes are already paired and condensed, the oocytes begin the diplotene step (Grier et al., 2007). Oogonia and newly formed primary oocytes present similar sizes (ranging from 4 to 15 μm), but can be discerned by microscopic observation. This is because in the spherical nucleus of the early oocytes in the diplotene step, the chromosomes can already be individualized and stain differently from oogonia (more details in Guraya, 1986; Grier et al., 2007; Lubzens et al., 2010; Cassel et al., 2017). Oocytes at this stage of development initiate the process of "primary growth," and in parallel with the changes that occur in the nucleus, several modifications also occur in the cytoplasms of these cells. Primary oocytes at the beginning of the diplotene step are cells with an electron-lucent cytoplasm, which has not yet acquired the basophilic character typical of the later stages of primary growth. In electron microscopy, these oocytes have cytoplasm with groups of mitochondria and nuages scattered near the nucleus. There is also the early development of small microvilli that extend from the oocyte toward the prefollicular cells, that is, the primitive zona pellucida (Cassel et al., 2017).

It is commonly accepted that, in the nucleus, the chromosomes stagnate in the diplotene step, at which point the follicle formation begins. At the beginning of the primary growth phase, the oocyte displays a scarce cytoplasm and central nucleus, containing a single nucleolus also central (Guraya, 1986). From that moment, the nucleus is called the germinal vesicle and

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the surrounding cells are called the follicular cells. These cells constitute, together with the oocyte, the initial ovarian follicle that remains connected to the germinal epithelium (Grier et al., 2007). This type of oocyte is also commonly described in the ovaries of neotropical migratory species (Lima et al., 1991; Romagosa et al., 1993a, 2002; Cassel et al., 2017; among others).

Primary "perinucleolar" oocytes

The stage of primary oocytes with "multiple nucleoli" is characterized by the observation of several central nucleoli in the germinal vesicle. This phase, however, is soon replaced by the following, in which the various nucleoli are displaced to the periphery of the germinal vesicle and characterize the perinucleolar oocytes.

The typical characteristic of perinucleolar oocytes is the intense cytoplasmic basophilia, which occurs due to an intense accumulation of RNA. During this stage, several structures of the follicle are directed toward the synthesis of the cytoplasm and its components. This intense cellular activity is evidenced by the proliferation, accumulation, and differentiation of various cytoplasmic structures such as free ribosomes, mitochondria, Golgi apparatus, lipid droplets, and other structures. An aggregate close to the nucleus in the young oocytes is formed, known as the Balbiani vitelline corpuscle. This aggregate contains membranous elements such as the endoplasmic reticulum, ribosomes, and mitochondria (details of the composition of this structure in Guraya, 1986; Cangussu-Mariani et al., 1990). At this stage, the oocytes often increase in volume, so the nucleus/cytoplasm ratio decreases. Upon reaching this size, the oocyte is surrounded by a zona pellucida and remains within its definitive follicle, consisting of a layer of internal epithelial cells (granulosa layer or follicular layer), a basal membrane, and, more externally, a layer of flattened squamous cells, the theca cells (Guraya, 1986).

Due to the presence of numerous nucleoli positioned at the periphery of the oocyte nucleus, the oocytes are referred to as in the perinucleolar stage, which is widely described in native migratory fish (Lima et al., 1991; Romagosa et al., 2002; Honji et al., 2009; and others). Until this stage of development, the oocytes are considered as previtellogenic and the ovaries that are filled by these cells are usually classified as "developing" (Narahara et al., 1988; Lima et al., 1991; Romagosa et al., 1993a; among others).

Vitellogenesis

After the accumulation of several types of RNA, organelles, and cytoplasmic inclusions that occurred in the previously mentioned phases (Fig. 14.4), cortical alveoli and two types of yolk granules are detected in the oocytes. In addition to yolk granules, glycogen may also be present. With continuous deposition of yolk, rapid oocyte growth occurs, which is accompanied by intense metabolic activity (Guraya, 1986). Due to the diversity of size of the vitellogenic oocytes, several nomenclatures and subdivisions are used. However, there is a striking similarity in the morphological descriptions of this process among the different species of native migratory fish analyzed. Several descriptions indicate the appearance of peripheral cortical alveoli in the oocytes as one of the most evident markers of the onset of vitellogenesis (Ganeco et al., 2001; Romagosa et al., 2002; Grier et al., 2007; and others).

Oocytes in early vitelogenesis (Fig. 14.4) may also be included in a phase widely called "secondary growth," which is applied to both native migratory species (Romagosa et al., 2002) and anadromous fish such as the common snook, *Centropomus undecimalis* (Grier, 2000). In the secondary growth stage, the oocytes transit between the endogenous vitellogenesis, sometimes characterized by predominant lipidic vitellogenesis (Honji et al., 2009), and the exogenous vitellogenesis, marked by the intense deposition of proteic yolk granules (more details in Guraya, 1986).

In native migratory species, ovaries containing this type of oocyte are often referred to as "spawning capable" ovaries (further details are given in Zaniboni-Filho and Weingartner, 2007; Cassel et al., 2017). Small differences can be found in this process, even among native species. It is worth emphasizing that particularities in the processes are important and may denote distinct physiological and reproductive characteristics among species that should be observed, mainly in those species that are to be reproduced in captivity (Bazzoli et al., 1996).

In this context, different biochemical compositions of the structures that form the cortical alveoli and the proteic yolk are found in native species, and these variations may be related to specific functions during oogenesis and fertilization (Bazzoli et al., 1996). However, only a few studies have investigated more specific aspects about the composition of oocyte reserve structures. As an example, in *Hypostomus* cf. *tientensis*, the adhesive oocytes display carbohydrates throughout all stages of development (Favaro and Chaves, 1999). Also, carboxylic acid carbohydrates were found only in the vitellogenic stages, which, according to the authors, may be necessary for the maintenance of oogenesis in this species.

Regarding the mechanisms that regulate and promote vitellogenesis, it is known that after primary growth, in response to environmental stimuli, periodic discharges of pituitary hormones into the bloodstream induce the onset of yolk

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production (vitellogenesis). Although the specific mechanisms of this process are not fully understood in native migratory species, some studies (Gazola et al., 1996; Barcellos et al., 2001; Jesus et al., 2017) show similarity with the processes described in species of temperate regions, especially in salmonids (revisions in Nagahama and Yamashita, 2008; Lubzens et al., 2010). In these species, the main trigger of the vitellogenic process is the FSH (Nagahama and Yamashita, 2008). This process is responsible for the great increase in ovarian weight in most species (up to 20% of total body mass), and in this period the ovaries are called mature. The vitellogenic phase is accompanied by intense metabolic activity, with rates of oxygen uptake and amino acid incorporation being increased many times during vitellogenesis (Guraya, 1986).

The vitellogenic phase also comprises the completeness of the development of the zona pellucida and the micropylar apparatus. The zona pellucida is a complex structure that consists of an extra-oocyte matrix in which there is the development of microvilli between the oocyte and the follicular cells. The production and organization of these microvilli support the development of heterologous communicating junctions that are required for physiological communication during oocyte maturation (Andrade et al., 2001; Lubzens et al., 2010). The micropylar apparatus is a concave region in the zona pellucida that tapers toward the plasma membrane. Covering this concavity, there is a highly specialized and hypertrophied cell in the follicular layer called the micropylar cell. This funnel-shaped apparatus closes after sperm passage, preventing polyspermy (Lubzens et al., 2010).

In neotropical species, with the advancement of vitellogenesis, cytoplasmic basophilia disappears completely, and the cytoplasm becomes intensely acidophilic with the homogeneous distribution of yolk granules. The layers surrounding the follicle are easily distinguishable, and the nucleus remains central or slightly eccentric (Honji et al., 2009). In this context, little is known about the specific functions of the reserve substances that compose the oocytes and their specific relationships with the different processes of embryonic and larval development. This subject is important for understanding the larvae production of native species in captivity, as it involves aspects such as the specific formulation of the feeds for matrices. Until now, to the extent of our knowledge, this subject has not yet been explored with the necessary depth.

In native migratory fish, after completion of vitellogenesis, the ovarian activity reduces and remains in tune with the adaptation to the environmental conditions. This is to ensure that the release of the oocytes coincides with the period in which these characteristics are more adequate, providing the ideal conditions for the offspring. At this stage, oocytes are classified as complete vitellogenesis or in a "dormancy period," and the duration of this period varies among species, from a few weeks to a few months (Zaniboni-Filho and Weingartner, 2007).

The morphological characteristics of oocytes in complete vitellogenesis are similar among species. In general, the main differences are related to the final diameter of the oocytes, the quantitative ratio between the different types of yolk, and the composition of the different yolk granules and cortical alveoli. Regarding the diameter of oocytes throughout their development, it has been reported that, for most characid species, the diameter of oocytes in complete or mature vitellogenesis (after GVBD) varies between 650–1500 μm (Winemiller, 1989). These values agree with those found for *A. altiparanae* and other species of *Astyanax* (Barbieri, 1992; Santos et al., 1996). In *A. altiparanae*, vitellogenic oocytes ranged from 634.5–685.0 μm in diameter (Cassel et al., 2017). For *Astyanax henseli*, the diameter of vitellogenic oocytes ranged from 800–1120 μm (Dala-Corte and Azevedo, 2010), and for *A. scabripinnis* it was 620.39 μm (Melo et al., 2005; Pereira Filho et al., 2011).

The presence of vitellogenic or mature oocytes is used in fish farming practices for the selection of reproducers. It is mainly based on oocyte diameter size (hence the importance of species-specific knowledge), germinal vesicle positioning, and the frequency of different types of mature oocytes, through specific techniques. The cannulation technique is the most accurate to correctly select suitable breeders (Schorer et al., 2016), but can be replaced using endoscopic analysis in species where there is no direct communication between the ovary and urogenital papilla by a duct, as in the case of *A. gigas* (Torati et al., 2016).

After the stage of complete vitellogenesis, the oocytes are ready for the next step of oogenesis, which is the resumption of meiosis (mature oocytes) and ovulation (Nagahama and Yamashita, 2008). Regarding the rheophilic neotropical species, when they are kept in captivity, they are not able to complete this process and are induced to spawn with hormonal treatments (Zaniboni-Filho and Weingartner, 2007). This subject will be covered in depth in another chapter of this book.

The final maturation and ovulation are relatively fast steps that occur at intervals of 6–40 h depending on the species, protocol, number of doses, type of hormone, type of system applied (seminatural spawning or extrusion), and other factors in neotropical migratory fish when subjected to hormonal induction in captivity. Most of the spawning descriptions were obtained under captive conditions through hormonal induction. Among the species already induced and studied, we can highlight the cachara (Leonardo et al., 2004; Figueiredo-Ariki et al., 2017), the curimbatá (*P. lineatus*) (Paulino et al., 2011; Hainfellner et al., 2012a; Andrade et al., 2014; Viveiros et al., 2015), the dourado (Sanches et al., 2009), the jaú (*Zungaru jahu*) (Nogueira et al., 2012), the jundiá (*R. quelen*) (Carneiro and Mikos, 2008), the lambari (*A. altiparanae*) (Felizardo

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et al., 2012), the matrinxã (Ramos et al., 1997; Dias et al., 2011; Hainfellner et al., 2012b; Zanuzzo et al., 2018), the pacu (Carolsfeld et al., 1988; Paulino et al., 2011; Kuradomi and Batlouni, 2018), the piapara (*Megaleporinus elongatus*) (Sato et al., 2001; Pereira et al., 2018), the piau (*Leporinus piau*) (Sampaio and Sato, 2009), the piau-de-vara (*Schizodon fasciatus*) (Lopes and Leal, 2010), the piauçu (*Megaleporinus macrocephalus*) (Pereira et al., 2017), the piracanjuba (Paulino et al., 2011), the surubim do Paraíba (*S. parahybae*) (Caneppele et al., 2009; Honji et al., 2013a,b; Okawara et al., 2015), and the tambaqui (Muniz et al., 2008; Acuña and Rangel, 2009; Souza et al., 2018), among dozens of others.

The oocyte maturation process, that is, the resumption of meiosis, occurs after vitellogenesis in mature oocytes and is characterized by drastic morphological changes (for a review, see Nagahama and Yamashita, 2008; Mylonas et al., 2010). In neotropical migratory fish, the oocyte maturation was described in some species that had their ovaries evaluated during the hormonal induction process. This process was characterized by the presence of oocytes with a diameter slightly superior to those of vitellogenic oocytes, migration and breakdown of the germinal vesicle, and fusion of the yolk granules (Leonardo et al., 2004) in the matrinxã (Hainfellner et al., 2012b), the pacu (Criscuolo-Urbinati et al., 2012), the piauçu (Pereira et al., 2017), and the piapara (Pereira et al., 2018). The almost complete absence of reports and descriptions of oocyte maturation and ovulation in migratory species in natural environments is expected because these processes are relatively rapid and occur at the end of the migration process, but mainly because interventions at this stage would certainly culminate in interruption or alteration of the reproductive process.

At ovulation, the follicular layer, the basal membrane, and the theca layer split in a coordinated manner, creating an opening through which the oocyte moves from the follicle to the ovarian lumen. The layers of cells remaining after the oocyte release form the postovulatory complex. Thus, information on spawning times of neotropical species (migratory or nonmigratory) in nature is usually restricted to general data from periodic collections and descriptions of the macroscopic and/or histological characteristics of the ovaries (Pereira et al., 2013; Normando et al., 2014, Brandão et al., 2017; and many others). The main spawning indicator is the presence of postovulatory complexes (detected histologically) remaining in postspawning ovaries (Ganias, 2012). However, the duration of the reabsorption process of postovulatory complexes is also usually rapid and difficult to identify in collections in the natural environment. In this way, the descriptions of the presence and the morphological and temporal characteristics of the resorption process of the postovulatory complexes are more common in captive hormone-induced spawning. In common snook, the process of disintegration of the basal membrane and disappearance of the postovulatory complexes was meticulously described as occurring in 48 hours postspawning (Grier et al., 2017). Few neotropical migratory species had the time of reabsorption of the postovulatory complexes established; in *P. fasciatum*, the process was followed for 24 h, during which an intense follicular reabsorption process was reported, but without its complete disappearance (Romagosa et al., 2005). In lambari (*Astyanax bimaculatus lacustris*), the reabsorption process was followed for 4 days, a period in which a reduction of the complexes was described, associated with an intense apoptotic process, but without complete reabsorption (Drummond et al., 2000).

Hormonal control of oogenesis

As already seen in this chapter, the pituitary produces two gonadotropic hormones, or gonadotropins (GtHs): FSH and LH, whose action targets are mainly in the gonads. According to Nagahama and Yamashita (2008), in salmon and for several other species of teleosts already studied (such as zebrafish, in Clelland and Peng, 2009), the growth and maturation of ovarian follicles is dependent on the dynamics of gonadal steroid secretion, which, in turn, are controlled by gonadotropins (Fig. 14.4). Thus, FSH acts in the oocyte growth phase, having a predominant role in regulating the growth of vitellogenic follicles, activating the production of E2 by the ovarian follicles themselves, and regulating ovary development through the control of the synthesis of vitellogenin. LH, in turn, acts on oocyte maturation by stimulating the secretion of 17α,20β-DHP, which has been identified as the maturation-inducing hormone (MIH) for most teleosts, and this latter induces the resumption of meiosis in oocytes with the consequent formation of the female gamete (Nagahama and Yamashita, 2008).

The way steroids are produced involves a complex cascade of specific enzymes present in the cell layers that cover the oocyte (Fig. 14.4). These layers, as in the other vertebrates, are the granulosa layer (or follicular layer), more internal, and the theca layer, more external, separated by a basal membrane. The process of steroidogenesis has been called the "two-cell type model" because it occurs in steps integrating these two layers. Thus, from cholesterol, which is the precursor of all steroid hormones, several steps take place until its conversion to testosterone, whose next step will give rise to E2 by the P450 aromatase enzyme. Also, from cholesterol, after several conversion steps, the enzymes P450c17 and 20β-HSD are required for the production of 17α,20β-DHP (reviews in Nagahama and Yamashita, 2008; Clelland and Peng, 2009).

The study of the steroid profile in females of neotropical teleosts has great importance today, especially regarding the migratory species that can have their migratory route interrupted by the presence of dams without effective mechanisms for

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the transposition of these fish. In this case, the study of plasma hormone levels of pituitary hormones, and especially gonadal steroids, would indicate the most active hormones in each situation, that is, the normal cycle and the confinement situation (Gazola et al., 1996; Amaral et al., 2007; Arantes et al., 2010). These studies can provide subsidies for each species in case of fish farming, helping to make breeding successful. Among the neotropical species studied in relation to the gonadal steroid profile, we can mention some, such as *A. altiparanae* (Jesus et al., 2017), *M. elongatus* (Pereira et al., 2018), *M. macrocephalus* (Pereira et al., 2017), *P. mesopotamicus* (Gazola et al., 1996; Schorer et al., 2016; Kuradomi and Batlouni, 2018), *Prochilodus argenteus* (Arantes et al., 2010), *P. lineatus* (Hainfellner et al., 2012a), *R. quelen* (Barcellos et al., 2001), and *S. hilarii* (Amaral et al., 2007; Moreira et al., 2015).

Furthermore, the analysis of gonadal steroids can support noninvasive studies that allow the determination of the sex of the animal whose species does not have sexual dimorphism. These results can be of great use in the practice of fish farming, as demonstrated by the study done with the pirarucu (Chu-Koo et al., 2009). In addition, we emphasize that the use of hormonal implants (GnRH, gonadotropins, or gonadal steroids), widely used in several fish farming species to synchronize spawning as well as anticipate and predict the breeding season (for review see Mylonas et al., 2010), has been recently tested for viability in native migratory species such as the pacu (Kuradomi et al., 2017) and has promising results.

Spermatogenesis

In the vertebrata, the spermatogenesis is a highly complex and conserved process that starts with diploid cell proliferations, marked by the multiplication of spermatogonia, followed by cellular modifications resulting in the activation of meiotic machinery and subsequent differentiation of haploid cells in spermatozoa.

On the other hand, when the spermatogenesis of mammalian and fish are compared, there are great differences. The mammalian Sertoli cells show a pyramidal shape and the plasmatic membrane shows invaginations where the germ cells are disposed. The spermatogonia are located at the basal-lateral surface while the other germ cells are distributed along the lateral surface, with the spermatids restricted in the upper side surface of the Sertoli cells. Thus, this organization is responsible for forming a stratified epithelium in the mammalian adult germinative epithelium of males.

In fish, the spermatogenesis is considered cystic because the Sertoli cells are responsible for supporting the germ line cells, which are surrounded by cytoplasmic processes of Sertoli cells forming structures known as cysts (Fig. 14.5). All processes of multiplication and differentiation of the spermatogonia up to the spermatozoa formation are supported and provided by the Sertoli cells. The size and volume of the cysts increase as the spermatogenesis occurs. This fact can be explained by the increase in the number of germ cells in the inner part of the cyst, due to sequential division processes experienced by these cells. The fish Sertoli cells, unlike those of mammalians, maintain the potential for mitosis (Nóbrega et al., 2009; Schulz et al., 2010). At the end of spermatogenesis, the cyst is opened, and the spermatids or spermatozoa are released into the tubule lumen (Fig. 14.5).

The spermatogonium is the cell of the testes responsible for originating all the other germ cells. Mitotic divisions of spermatogonia occur both for self-renewal of spermatogonia and for the spermatogenesis compromised spermatogonia. So, throughout the mitotic division, the *undifferentiated** (spermatogenesis compromised spermatogonia) and *undifferentiated* (self-renewal of spermatogonia) type A spermatogonia undergo multiplications and, after some morphological changes, originate the *differentiated type A* spermatogonia. Meanwhile, the differentiated type A spermatogonia do not have self-renewal potential and give rise to type B spermatogonia. The amount of mitosis of each type B spermatogonium is characteristic of the species, and in the inner part of one cyst, cytoplasmic bridges connect the daughter cells to each other (Schulz et al., 2010). By a new differentiation process, the type B spermatogonia become type I spermatocytes that begin the first meiotic division, in which the resulting cells are type II spermatocytes. Finally, these cells finish the first meiotic division, promoting the second meiotic division, and give rise to spermatids. After the process of differentiation, known as spermiogenesis, the spermatids originate the spermatozoa.

The size and volume of the germ cells are characteristic of the species, but throughout spermatogenesis, these cells decrease their size as well as the size of the nuclei, and the spermatozoa are the smallest ones. Dias et al. (2017) show that, in *P. lineatus*, the cell volume and nuclear volume in the type A spermatogonia are 1252 μm^3 and 413 μm^3, respectively. These authors also reveal the decrease in the size and volume of the germ cells as well as the nuclei throughout the process of spermatogenesis. Camargo et al. (2017) show that the reduction process of the length of the germ cells can occur in the differentiation of the type A spermatogonia. The authors show that, in *A. altiparanae*, the major length axis of the undifferentiated* type A is 19,707 μm while the undifferentiated type A is 16,975 μm.

In fish, the process of spermiogenesis can occur when the spermatids are in the inner part of the cysts, which is called cystic spermiogenesis, or it can occur when the spermatids are in the lumen tubule, and in this case, the spermiogenesis is

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called semicystic. Additionally, the lumen of the tubule can be fulfilled by a seminal secretion such as found in *A. altiparanae*, a species of Characiformes that shows a semicystic spermiogenesis process (Costa et al., 2014).

The position of the germ cells in the germinative epithelium of fish has been highly studied (Nóbrega et al., 2010, 2015; Schulz et al., 2010; Camargo et al., 2017). In *A. altiparanae*, it was demonstrated that this species also has two types of A spermatogonia, and the undifferentiated* type A spermatogonia are located near the interstitial compartment while the undifferentiated type A spermatogonia are concentrated in the intertubular area (Camargo et al., 2017).

Morphology of testis

The testis of fish is generally a paired organ, elongated, and enveloped by the albuginea, a capsule of connective tissue. Reflecting several reproductive strategies and different stages of the gonadal cycle, the testes of fish can show variations in size, weight, color, and shape.

Among the South American teleost fish, the majority show smooth testes,such as *A. altiparanae* (Costa et al., 2014). However, the fish belonging to the Siluriform group generally show a great number of fringes, such as can be observed in *Iheringichthys labrusus* (Santos et al., 2001), *P. fasciatum* (Batlouni et al., 2006), and *Lophiosilurus alexandri* (Barros et al., 2007).

Concerning the organization of the seminiferous tubule, the testis of fish can be classified as anastomosing tubule testis or lobular testis. In the anastomosing tubular type, the seminiferous tubule interconnects, forming a highly branched and anastomosing network. The lobular type has a seminiferous tubule that terminates blindly in the periphery of the organ, below the albuginea tunica. It can be divided into two subtypes, the spermatogonial unrestricted and the spermatogonial restricted type. The spermatogonial unrestricted type is characterized by the presence of spermatogonia along all the lobules. On the other hand, the spermatogonial restricted type has the spermatogonia only in the distal part of the tubule, located near the albuginea tunica area (Grier, 2002).

Nevertheless, in the literature, different forms of descriptions of the testis of fish can be found. For example, Vicentini et al. (2002) show the morphology and the ultrastructure of the testis of *P. scrofa*, describing that the seminiferous tubule of this species is anastomosed and has an unrestricted distribution of spermatogonia along the germinative epithelium.

The histological analysis of fish testis as well as in other vertebrates allows recognizing two compartments: the germinative epithelium and the interstitial compartment, separated by a basement membrane (Grier, 2002). In the germinative compartment, two groups of cells can be seen: the germ cells and the Sertoli cells. When the Sertoli cells envelop the germ cells, they form the cysts. The interstitial compartment is composed of cells of the connective tissue, smooth muscular fibers, Leydig cells, and peritubular myoid cells.

Costa et al. (2014) demonstrated that, in *A. altiparanae*, the Sertoli cells, besides supporting the germ cells, play another role and acquire a secretory function. These authors showed that at the beginning of the reproductive cycle of *A. altiparanae*, the Sertoli cells support spermatogonia and the other germ cells. After the release of spermatids, the Sertoli cells undergo changes in their shape and promote a secretory function (Fig. 14.5).

Myoid peritubular cells are found below the basement membrane of the germinative epithelium. These cells are elongated and have contractile activity, which helps in the displacement mass of spermatozoa along the tubule lumen. Additional functions have been proposed for the myoid peritubular cells of mammalians, such as the production of the extracellular matrix compounds, secretion of growth factors, and participation of the hematotesticular barrier. In fish, other functions of these cells are yet unknown.

The Leydig cells (Fig. 14.5) are involved in the synthesis of steroid hormones, a function that is better studied in the mammalian testis. In fish, the Leydig cells are found in the angular interstitium, that is, in areas not occupied by the seminiferous tubules, where the interstitial tissue is larger. Nóbrega and Quagio-Grassioto (2007) demonstrated the presence of the 3β-HSD enzyme in the Leydig cells of *S. spilopleura*. This enzyme is essential to the biosynthesis of steroid hormones, which corroborates the attribution of steroidogenic function to these cells in fish.

Male reproductive cycle

Most of the teleosts show morphological changes in the testis along the reproductive cycle, and the histological characteristics can be used to classify the stage of the reproductive cycle. Currently, this is the most secure method to reveal the stage of the reproductive cycle of fish testis, despite several other methods used within the past 50 years to classify the reproductive cycle of these animals.

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One highly used method was the gonadosomatic index. Despite its wide use, this method is not completely able to show the modifications of the several stages of maturation of the fish testis because these changes may not be reflected in the weight of the organ, and this can cause mistakes in the interpretation of the data.

Godoy (1975) proposed a scale of macroscopic maturation of male and female gonads of *P. scrofa*, with 10 stages that consider the color, the weight, and the presence or absence of spermatozoa. After that, histological analysis of the morphological changes of the fish testis was used to determinate the stages of the reproductive cycle using different terminologies to determine the stages. For example, Barbieri et al. (1981) described the anatomy and histology of the testis of *Geophagus brasiliensis*, classifying the stages of the reproductive cycle in virgin or immature as well as in maturation, mature, and empty. Andrade and Godinho (1983) described the reproductive cycle of males of *Leporinus obtusidens* (*Leporinus silvestrii*), classifying four stages called resting, in maturation, mature, and empty. One year later, Barbieri and Barbieri (1984) described the morphology and histology of the testis of *Gymnotus carapo,* also classifying the reproductive cycle in four stages but replacing the term "empty" with "spent."

Romagosa et al. (1993b) showed the morphological changes of the testis of *P. mesopotamicus* in a captive situation, also using different terminologies to classify the stages. For this species, the last stage of the reproductive cycle of the testis was considered regressed. The authors justified that in the testis of *P. mesopotamicus*, after the mature stage, in captivity, spermiation does not occur, but the size of the testis decreases and there is also a disorganization of the seminiferous tubule.

The method proposed by Grier (2002) focuses on the histological analyses of the germinative epithelium, considering the observation of the continuous or discontinuous epithelium. The germinative epithelium is considered continuous when it is surrounded by Sertoli cells associated with germ cells forming the cysts. On the other hand, when the cysts are opened and the germ cells are released in the tubule lumen, with the epithelium showing areas that have only Sertoli cells, this characterizes the discontinuous epithelium.

Batlouni et al. (2006) used the method proposed by Grier (2002), then characterized the five stages of the reproductive cycle of male *P. fasciatum*. They showing that the germinative epithelium is continuous at the initial and mid maturation as well as regressed while at the final maturation and regression, the germinative epithelium is discontinuous. Costa et al. (2014), using stereological and morphometric approaches in a quantitative form, revealed the histological changes of the testes of *A. altiparanae* throughout the reproductive cycle, showing the relative proportion of interstitial and germinative compartments.

Despite the high frequency of teleost species that show morphological changes of the testis during the reproductive cycle, there are species that do not show these modifications. For example, Nóbrega and Quagio-Grassioto (2007) demonstrated that the testes of *S. spilopleura* have two regions called cortical and medullar, which remain constant throughout the year. The medullar region shows a continuous germinative epithelium and is involved in spermatogenesis while the cortical region exhibits dispersed cysts with the predominance of Sertoli cells and is a storage place for spermatozoa.

Structure of the spermatozoa

Spermiogenesis is the process of the differentiation of spermatids in spermatozoa. Throughout this event, remarkable morphological changes occur in these cells, such as centriole migration, flagellum formation, condensation of the chromatin, formation of the nuclear fosse, and migration of mitochondria to the intermediate piece.

The spermatozoa shapes of different teleost species can show morphological changes, but generally they have three common structures: the head where the nucleus is located; the middle piece that contains mitochondria; and the flagellum, whose number is characteristic of each species. Usually, the spermatozoa of teleosts do not show the acrosome, and this structure seems unnecessary to perforate the oocyte because of the presence of the micropyle in the oocytes as a specific place where spermatozoa can penetrate the oocyte.

As mentioned before, most teleosts display cystic spermatogenesis, where the differentiation of the spermatid into spermatozoa occurs within the cysts, and when the junctions between Sertoli cells are broken, the spermatozoa are released on the tubule lumen. On the other hand, there are species where the spermiogenesis is classified as semicystic and, in this case, the spermatids are released from the cysts, and the spermiogenesis is concluded in the tubule lumen.

Gusmão et al. (1999) described the ultrastructure of the spermiogenesis of *Plagioscion squamosissimus*, showing that the spermatozoa of this species have a round head with one flagellum positioned parallel to the basis of the nucleus. The mid piece has a necklace of mitochondria that remains separate from the flagellum by membranes, forming a structure known as the cytoplasmic channel. Spermatozoa that show this shape are classified as perciforms because they are typical of this order.

Spermatozoa of *Iheringichthys labrosus* have a morphology like that described for Perciformes; however, in this species, the flagellum is perpendicular to the basis of the nucleus (Santos et al., 2001). In the same way, spermatozoa

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of *B. cephalus*, described by Romagosa et al. (1999), are like those of *I. labrosus*, but the basal body of the flagellum is inserted into a cavity in the nucleus, known as nuclear fosse.

Spermatozoa with pointed heads that contain two flagella are found in *Lepidosiren paradoxa* (Matos and Azevedo, 1989). In the proximal portion of the flagellum, there are lateral expansions of the cell membrane. These expansions are found in some species of fish and produce a highly efficient movement of the flagellum and displacement of the spermatozoa in the water.

Species of fish that have internal fertilization show some differentiation in their testis, and they form structures composed of spermatozoa clusters that are known as espermatozeugmata. Spermatozoa packaging can bring a greater efficiency in the transfer of male gametes to the female reproductive tract. In all the Poiciliidae that have internal fertilization, the elongated head of the spermatozoa, into the cysts, is positioned in cytoplasmic recesses of Sertoli cells. A t the end of spermiogenesis, the cysts are opened and the spermatozeugmatas are released into the tubule lumen (Grier, 1993).

Hormonal control of the spermatogenesis

The control of spermatogenesis is a complex process in which the gonadotropins (LH and FSH), androgens, and growth factors are involved. The main androgen of teleosts involved in the spermatogenesis stimulation is the 11-ketotestosterone (11-KT).

FSH acts on the stimulation of the proliferation of spermatogonia in the initial stage of spermatogenesis. Ohta et al. (2007) demonstrated that the main effect of FSH in the Japanese eel (*Anguilla japonica*) is to stimulate spermatogenesis, mediated by androgen production (11-KT). These authors also showed that the Leydig and Sertoli cells surrounding type A spermatogonia and early type B spermatogonia express the eel FSH receptor. Thus, one mechanism to promote spermatogenesis in fish is by stimulating androgen production by FSH.

Crespo et al. (2016) investigated the influence of FSH as well as genes/pathways genes involved in the spermatogenesis of zebrafish, demonstrating that FSH-stimulated spermatogonial proliferation was accompanied by modulation of several signaling systems (Tgf-β, Hedgehog, Wnt, and Notch pathways). These authors showed that FSH modulates the expression of approximately 200 genes in a steroid-independent manner.

In the Japanese eel, it was demonstrated that the anti-Müllerian hormone (AMH), which is known to avoid the development of females in mammalians, stimulates the proliferation of spermatogonia and, at same time, inhibits the differentiation of spermatogonia. By contrast, the proliferation of spermatogenesis-compromised spermatogonia is stimulated by 11-KT. The presence of this androgen stimulates the Sertoli cells to produce activin B, and at the same time, downregulates the expression of the AMH gene. These data connect the meiotic phase of spermatogenesis to 11-KT (Ohta et al., 2007)

Currently, other molecules related to spermatogenesis have been the target of investigations. The relation of Igf3 and AMH was studied in zebrafish by Morais et al. (2017). The researchers demonstrated that these growth factors act as antagonistics. Igf 3 is a fish-specific member of the Igf family. The Igf3 has a stimulatory effect in the proliferation and differentiation of spermatogonia, and the AMH, independent of androgen, is able to decrease the Igf3 transcript level. Therefore, the authors suggested that AMH is an important protein that plays an inhibitory role in the regulation of spermatogenesis, and that FSH stimulates the spermatogenesis and controls the inhibitory effects of AMH.

The control of the activation of fish spermatozoa is also the subject of studies. The maturation of Japanese eel spermatozoa is regulated by 17α,20β-dihydroxy-4-pregnen-3-one, the same progestagen that acts in females. In the male Japanese eel, this hormone is responsible for activating the carbonic anhydrase enzyme that is present in the spermatozoa membrane. The activation of this enzyme is responsible for the pH elevation of the seminal plasma and, at the same time, the elevation of cyclic AMP in the cytoplasm of these cells, which culminates in the activation of the spermatozoa (Miura and Miura, 2003; Schulz et al., 2010).

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Biology and Physiology of Freshwater Neotropical Fish

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