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Ovary Differentiation and Activity in Teleostei Fish

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Additional information is available at the end of the chapter

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Abstract

Teleostei fishes constitute a very large group among the vertebrates. They present several reproductive strategies, and many species are gonochoristics. During the gonadal differentiation, the gonadal primordium undergoes morphological changes giving rise to male or female gonads. Considering the lack of information about gonadal morphogenesis in Teleostei, especially in tangent aspects concerning the establishment of the germinal epithelium and its relation with the formation of the ovarian cavity, *Tanichthys albonubes*, *Corydoras schwartzi*, and *Amatitlania nigrofasciata* were taken as biological models to establish a comparative analysis of the female gonadal differentiation. In undifferentiated gonad, the epithelial cells associate with primordial germ cells and form germline cysts. These are distributed throughout the gonadal tissue; after the entrance of the oogonia into meiosis, the folliculogenesis occurs forming the first follicles, in a quite conserved process. However, the formation of the ovarian cavity is distinct. In *T. albonubes* and *A. nigrofasciata*, the lumen is formed by pleating and in *C. schwartzi*, it is formed by cavitation. The central lumen formed characterizes the cystovarian of Teleostei. Although there are differences in the chronology of the differentiation, the processes involved are quite similar and culminate in the formation of analogous structures.

Keywords: germinal epithelium, gonadal differentiation, germline cysts, folliculogenesis, cystovarian formation, Teleostei fish

1. Introduction

Teleostei fish represents about 50% of the vertebrates [1]. The bony fishes, within the Teleostei, are divided into Ostariophysi, Protacanthopterygii, and Neoteleostei.

Ostariophysi is the second largest superorder of fish, and it is considered the most basal among the Teleostei, representing about three quarters of the world's freshwater fish [1]. This

diverse group contains important fish in the area of feeding, sport fishing, aquarium, and research, such as the common carp and zebrafish (Cypriniformes), the characids and tetras (Characiformes), the catfishes (Siluriformes), and the electric eels (Gymnotiformes) [1, 2].

The Neoteleostei is another large clade of bony fish that includes most derived species among Teleostei, and it is also very important in several areas. Among the Neoteleostei, the most relevant group is the Perciformes, which presents the greatest diversity among all orders of fish, being the largest order among vertebrates [1]. The most popular Perciformes are the cichlids, such as tilapia.

Regardless of their position on the phylogenetic scale, in most Teleostei species, the reproduction is cyclic and seasonal, determining a series of morphophysiological modifications in their gonads [3]. Teleostei present several reproductive strategies [3, 4]. Among these, there are mechanisms of release of gametes in the aquatic environment for external fertilization, development of specialized organs for internal fertilization, posture of fertilized eggs after internal fertilization, and even internal gestation of the embryos [5].

In Teleostei, the sexual determination and gonadal differentiation are controlled by genetic, physiological, and behavioral factors [6]. The genetic sex of the embryo is determined at the time of the fertilization by the combination of the chromosomes from the male and female gametes, and sexual determination is defined as the sum of the genes responsible for the formation of the gonads and their characteristics [7, 8]. In this aspect, the genetic control is one of the main determinants of the gender, even though environmental factors, such as temperature, photoperiod, or salinity, also have a great influence on the process, determining the physiological gender of the fish [9].

Most Teleostei are dioecious or gonochoristic, that is, they present individuals with separated sexes. These fishes may present two types of gonadal development, classified as undifferentiated or differentiated gonochoristics. In undifferentiated gonochoristics, the undifferentiated gonad begins its development resembling an ovary. Subsequently, part of the individuals becomes male, while another part remains female. This natural condition is known as juvenile hermaphroditism. In the differentiated gonochoristics, the gonad differentiates directly in a testis or in an ovary [6, 10].

However, at the beginning of embryogenesis, the gender of the fish is not morphologically defined, since it does not have gonads differentiated in testes or ovaries, and there is no other developed characteristic which is associated to the reproductive system. There are only embryological precursors that will give rise to the ovaries and testes: the primordial germ cells (PGCs) and the somatic cells. At this stage of development, these cells are totipotent, and they can give rise to male or female gonads [6]. At some point during gonadal development, through hormonal chemical signaling, the gonad differentiates into the ovary or testis. Once this occurs and the gonadal tissue completes its differentiation, the fish becomes physiologically male or female [11].

The gonadal differentiation in the Teleostei includes changes in both somatic and germ cells [9], as mitotic divisions of oögonia or spermatogonia from the primordial germ cells, to

structural changes, including mitotic proliferation of the somatic cells [12]. As a result, there is the formation of the ovarian cavity and spermatic ducts and lobules that will give origin to the ovaries and testes, respectively [9, 13].

In males, it is known that primordial germ cells establish specific positions, depending on the pattern of testicular organization [14]. This pattern, found in adult males, differs between basal (Ostariophysi) and derived taxa (Neoteleostei) [15]. However, the same does not happen to adult females. In this aspect, this chapter will describe the gonadal morphogenesis, with special attention to the formation of the ovarian cavity and establishment of the germinal epithelium, in basal taxa (*Tanichthys albonubes* and *Corydoras schwartzi*) and derived taxa (*Amatitlania nigrofasciata*), verifying possible distinctions or existing patterns along gonadal differentiation and considering the position of the species on the phylogenetic scale. The different methods used for these analyses are described in “complementary material,” at the end of the chapter.

These three representatives were chosen because they are small ornamental species, quite resistant and known in ornamental aquarium. In addition, they can be reproduced in aquarium, presenting a fast period of differentiation. Although gonadal differentiation is quick in these representatives, the data showed here can be extrapolated to the species of their groups, since there is no difference in the events along the differentiation. Thus, despite the time of differentiation being species specific, the events and morphological changes are the same [16].

T. albonubes is from China, and it prefers low temperatures. It is one of the smallest known Cypriniformes (3–4 cm) [17]. Among the Ostariophysi, the Cypriniformes were chosen because they are the most basal order, that is, they represent the most basal taxa. *C. schwartzi* is a tropical fish, from South America [17], very important in the aquarium hobby. It is a small catfish (7 cm), and for this reason, it was chosen to represent the other catfish. Catfishes present considerable commercial importance, and many of the largest species are farmed or fished for food. *A. nigrofasciata* is popularly known as acara cichlid. It attains the maximum length of 10 cm. Like other cichlids, it is aggressive and territorialist. Originated from Central America, they prefer alkaline and hot water [17]. Here, this species represents all other cichlids, that is, the most derived taxa.

2. The ovary differentiation in Teleostei

2.1. Gonadal primordium

In Teleostei, as in other vertebrates, the primordial germ cells (PGCs) differentiate from yolk sac cells, originating from extragonadal regions, and migrate to the genital ridge during embryonic development [18, 19]. The genital ridge is formed by a thickening of the intermediate mesoderm, which protrudes ventrally into the coelomic cavity of the embryo being delimited by a mesothelium [6, 18, 19].

The primordial germ cells (PGCs) migrate to the genital ridge and are disposed between the somatic cells. Both cell populations proliferate by mitosis constituting the gonadal primordium [14, 16, 19, 20], as observed in *T. albonubes* (**Figure 1**). Therefore, during the morphogenesis period, the gonadal primordium is formed; it increases in length by mitotic proliferation of its cells, and it gives rise to an undifferentiated gonad [9, 10, 12, 14, 16, 21]. Now, the undifferentiated gonad will undergo a series of structural modifications determining the formation of a female or male gonad.

The gonadal primordia of the species here taken as representatives of the basal (*T. albonubes* and *C. schwartzi*) and derived taxa (*A. nigrofasciata*) present patterns of organization, which are very similar to each other and to the other Teleostei [22]. They are presented in pairs on the peritoneum along the coelomatic cavity, connected by a thin layer of gonadal mesentery. Each gonadal primordium is located ventrally to the kidney and dorsally to the swim bladder. The gonadal primordium extends throughout the entire coelomatic cavity, from the posterior to the anterior region (**Figure 1A and B**).

These characteristics of the gonadal primordium are also found in the adult form of the species that present an odd gonad, such as Poeciliids, a viviparous species, considered derived taxa [14, 23, 24]. In these, the formation of bilateral gonadal primordia is common. However, both gonadal primordia merge during the development of the gonadal tissue, forming a single organ in the adult individual [3, 14, 23].

Histologically, the gonadal primordia are formed by primordial germ cells (PGCs) and somatic cells. The somatic cells show varied forms, being predominantly squamous, with basophilic nucleus and scarce cytoplasm. The PGCs are large oval cells with voluminous nucleus and show quite evident nucleolus. Their cytoplasm is scarce and rich in "nüage," presenting positive response to metanil yellow, indicating the presence of proteins in its constitution (**Figure 1C**).

The primordial germ cells (PGCs) are distributed along the gonadal primordium, which is filiform, long, and thin, composed by only one or two layers of PGCs. Mitotic activity of PGCs may be occasionally visualized.

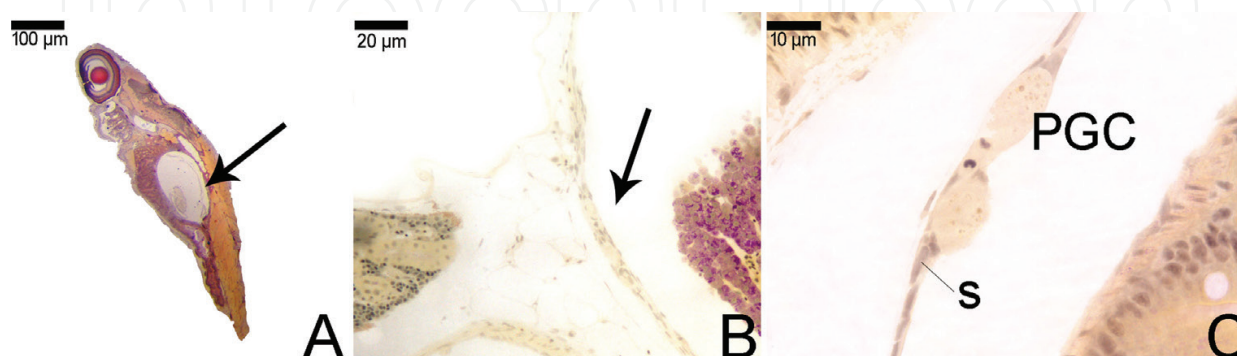


Figure 1. The gonadal primordium in *T. albonubes*. Light microscopy. Parasagittal sections. (A, B) The gonadal primordium (arrow) is located ventral to the kidney and dorsal to the gut. (C) The gonadal primordium is formed by primordial germ cells (PGCs) and somatic cell(s). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

At this stage, in any of the species, there are no morphological differences which suggest the evolution toward a male or female gonad. The number of germ and somatic cells gradually increases throughout the gonadal development. As a result, there is an increase in the size of the gonadal tissue, which becomes an undifferentiated gonad.

2.2. Undifferentiated gonad

The undifferentiated gonads of *T. albonubes* and *A. nigrofasciata* (Figure 2A–D) were observed in animals up to 30 days postfertilization (dpf) with 0.5 and 0.7 cm in length, respectively. In *C. schwartzi* the gonads remain undifferentiated (Figure 2E and F) for a longer period, up to 120 dpf, when the animals measure 2.5 cm.

In these species, as in most Teleostei, the undifferentiated gonads are pairs, long, and thin, occupying two-thirds of the coelomatic cavity from the urogenital papillae. They are formed by primordial germ cells (PGCs) dispersed among somatic cells.

In parasagittal sections, the undifferentiated gonads are thicker in comparison with the gonadal primordium, mainly due to the greater amount of somatic cells, which present irregular and squamous forms. The primordial germ cells (PGCs) remain in small numbers and are initially isolated between somatic cells, scattered throughout the gonad (Figure 2).

Since the undifferentiated gonads are formed only by primordial germ cells and somatic cells, they are very similar in any group of fishes, from the basal to the most derived taxa, including primitive fish such as sturgeon [25] or species with indirect gonochoristic development as Cypriniformes *Danio rerio* [26] and Characiformes *Gymnocorymbus ternetzi* [21], both Ostariophysians.

2.3. Gonadal differentiation

Morphological changes in the gonadal tissue, such as the formation of the ovarian cavity and the entrance into meiosis of the germ cells in females or the formation of the testicular ducts and lobules in males, are the main parameters used for the sexual distinction of gonochoristic gonads. Although these characteristics gather the consensus among different authors [6, 13], who use them as parameters for gonadal differentiation, the distinction between the female and male gonads may be detected prior to the entrance of the primordial germ cells into meiosis or before the formation of gonadal structures by the somatic cells. This detection of presumed female or male gonads is possible when considering the organization of germ and somatic cells in the gonadal tissue [14]. In other words, the gonadal differentiation in many species of Teleostei is closely related to the organization of the cellular types, which constitute the early gonadal tissue [14, 16].

A peculiarity observed here in *A. nigrofasciata* is the fact that the pattern of cellular organization in the undifferentiated gonads is different between female and male gonads [14], i.e., early gonads that will supposedly give rise to the ovaries and testes already present certain morphological differentiation. The same was observed in other derived taxa, such as *Poecilia reticulata* [14], being a common feature among the derived groups, that is, Neoteleostei.

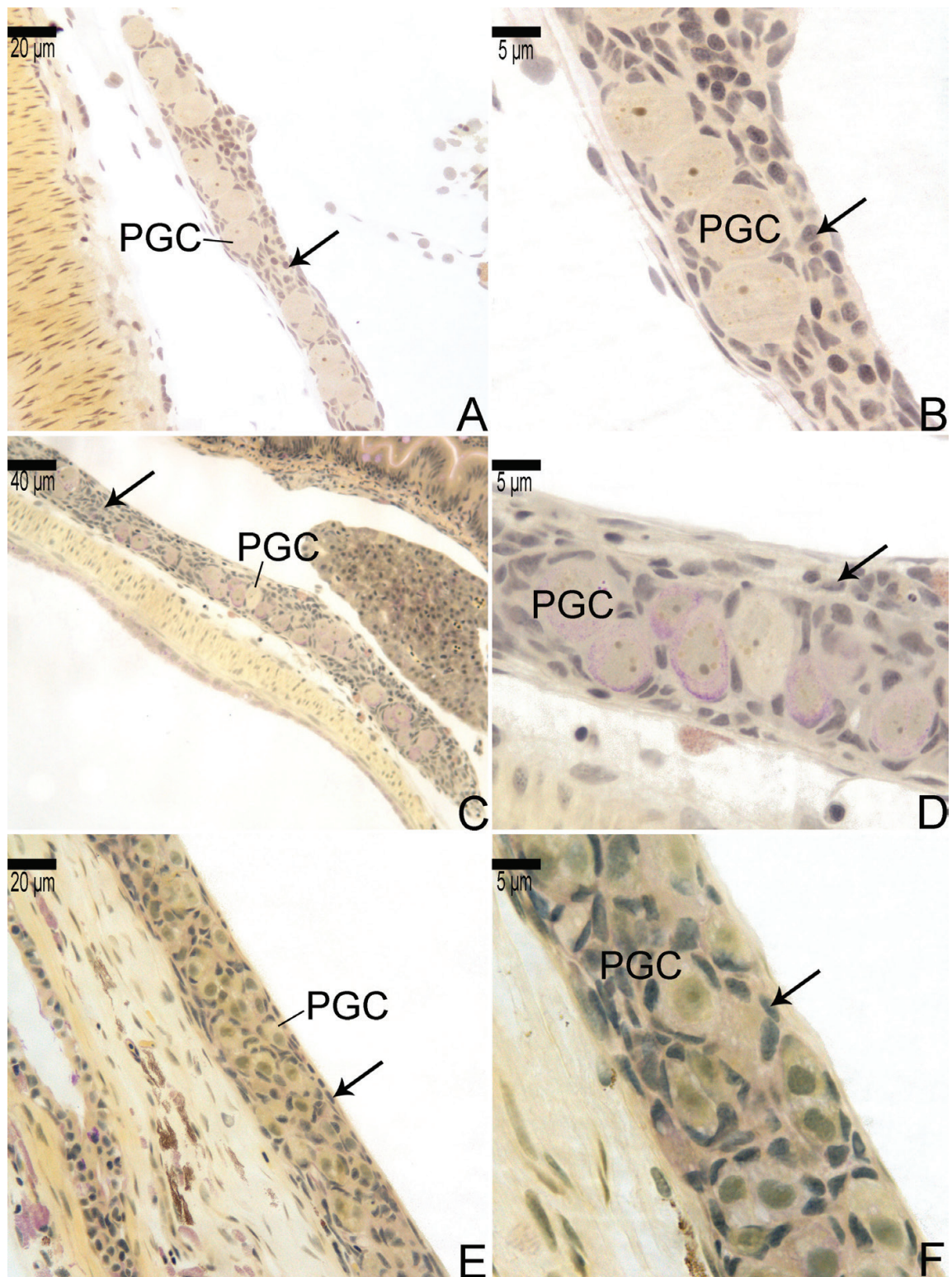


Figure 2. The undifferentiated gonads in *T. albonubes* (A, B), *A. nigrofasciata* (C, D), and *C. schwartzi* (E, F). Light microscopy. Parasagittal sections. The undifferentiated gonads are very elongated, are thin, and show a major number of somatic cells (arrow). The primordial germ cells (PGCs) are surrounded by somatic cells. Staining: periodic acid Schiff + hematoxylin + metanil yellow.

However, this pattern of organization of the Neoteleostei differs from the one found in the representatives of the Ostariophysi here utilized (*T. albonubes* and *C. schwartzi*), which present patterns similar to most of the existing descriptions for Teleostei [6, 12, 16, 18, 19, 22, 27].

In these, the supposedly female animals present primordial germ cells distributed in the central region of the early gonadal tissue, which has a smaller number of somatic cells concentrated mainly in the peripheral region of the gonad. At this stage, among most basal fish, it is possible to differentiate female from male gonads. In female gonads, the oogonia proliferate, form continuous cords of cells, and enter into meiosis, originating the first oocytes [16], while in male gonads, spermatogonia are organized in acinar structure or cell clusters, after forming continuous cords [14].

Thus, before the appearance of structures such as the ovarian cavity formation, the female gonadal differentiation in both Ostariophysi and Neoteleostei is initially marked by the appearance of meiotic figures in gonadal tissue [12, 13, 16].

The gonads of *T. albonubes*, *C. schwartzi*, and *A. nigrofasciata* differentiate directly into the ovary or testis, presenting direct gonochoristic development. In these three species, gradually and close to the period preceding gonadal differentiation, there is a small difference in the distribution of primordial germ cells (PGCs) along the gonadal tissue, between the supposedly female and male gonads.

In *T. albonubes* and *A. nigrofasciata*, the ovarian differentiation precedes the testicular differentiation and occurs around 37 and 120 dpf, respectively (in animals measure 1 and 3 cm). In contrast, in *C. schwartzi*, the ovarian and testicular differentiation occurs simultaneously around 130–150 dpf (3–4 cm).

In *T. albonubes* and *A. nigrofasciata*, the supposedly female gonad is smaller in size than the supposedly male one. The ratio of primordial germ cells (PGCs) to somatic cells is more balanced in females, whereas in supposedly male gonads, PGCs are scarce and are scattered among countless somatic cells. As a consequence, the male gonad becomes thicker than the female gonad [14]. Furthermore, in the supposedly female gonads, there is usually only a single line of PGCs delimited by somatic cells due to the gonad lower thickness.

In *C. schwartzi*, the first indication of gonadal differentiation refers to the organization of germ cells in supposedly male and female gonads. In female gonads, the oogonia form small germ-line cysts, separated by a highly developed interstitial tissue.

As it can be observed in these species, as well as in most Teleostei, the events involved in ovarian differentiation are quite similar, distinguishing between the species only in the chronology and the way in which the processes concurs to achieve the same final result—the formation of a cavity organ delimited by a germinal epithelium. Thus, the first stages of ovarian differentiation, characterized by the entrance of the germ cells into meiosis and the beginning of the folliculogenesis process, did not present significant differences between the species.

2.4. First folliculogenesis

The gonadal tissue of the analyzed species is thin, elongated, and formed by primordial germ cells (PGCs), now differentiated into oogonia, and somatic cells. The oogonia, immersed within the gonadal tissue, may be associated with somatic cells or remain isolated (**Figure 3A**). Isolated oogonia proliferate by mitosis giving rise to new oogonia (**Figure 3B**). When associated with somatic cells, they form a cyst of oogonium (**Figure 3C and D**), which originates the initial prophase oocytes, upon entering into meiosis, analogous to what occurs in the germinal epithelium of the ovigerous lamellae in sexually adult females [28–30]. The development of germ cells within each cyst is synchronous, due to the presence of cytoplasmic bridges between oogonia (**Figure 3E and F**) and prophase oocytes [16, 22, 31]. Thus, the cytokinesis is incomplete.

Since each oogonium gives rise to a cyst and the cellular divisions begin, different cysts are formed next to each other, giving rise to cell clusters, delimited gradually by a sole basement membrane in formation (**Figure 3C and D**). Thus, throughout the gonad, it is possible to observe individual isolated oogonia between somatic cells and cysts delimited by somatic cells derived from the epithelium, containing oogonia and/or early prophase oocytes (**Figure 3G**).

The oogonia are oval cells that present scarce cytoplasm with granulations corresponding to “nüages.” Their nuclei are large and spherical with one or more evident nucleoli. Its cytoplasm presents spherical mitochondria with tubular ridges, often associated with “nüages” (**Figure 3A–D**).

Oocytes present in the cysts are also rounded, with nuclei containing chromatin in different forms of organization according to the stage of the prophase in which they are found (**Figure 3G–M**), but their cytoplasm does not differ them, always remaining slightly acidophilus and scarce. Initially, the prophase oocytes have a more basophilic nucleus than the oogonia, and there is a decrease in the amount of “nüage” in the cytoplasm. The leptotene oocyte shows a strongly basophilic nucleus, with at least one nucleolus quite evident. With the progression of the prophase, the oocyte gradually lost nuclear basophilia. The zygotene oocyte presents greater chromosome condensation, giving the nucleus a granular aspect. Formation of the synaptonemic complexes begins, allowing the pairing of the homologous chromosomes. In pachytene, the synaptonemic complexes are totally formed. In the nucleus of the oocyte, there is a strong basophilia next to the nuclear envelope. These stages are illustrated below.

Now, the germline cysts containing diplotene oocytes are invaded by somatic epithelial cells—the pre-follicle cells (**Figure 3I–K**). Pre-follicle cells strongly united by numerous desmosomes complete and gradually involve each oocyte which separates from the cyst, giving rise to an ovarian follicle (**Figure 3L**). During this process, known as folliculogenesis, pre-follicle cells begin to form the basement membrane, after differentiating into follicle cells (**Figure 3M and N**). Gradually, the basement membrane is synthesized, individualizing each ovarian follicle. After the oocyte enter and remain in diplotene stage, the lampbrush chromosomes become visible. The cytoplasm of the oocytes increases, becoming gradually more basophilic and initiating the primary growth while within the germline cysts. Now, the diplotene oocyte isolated in the ovarian follicle, and in

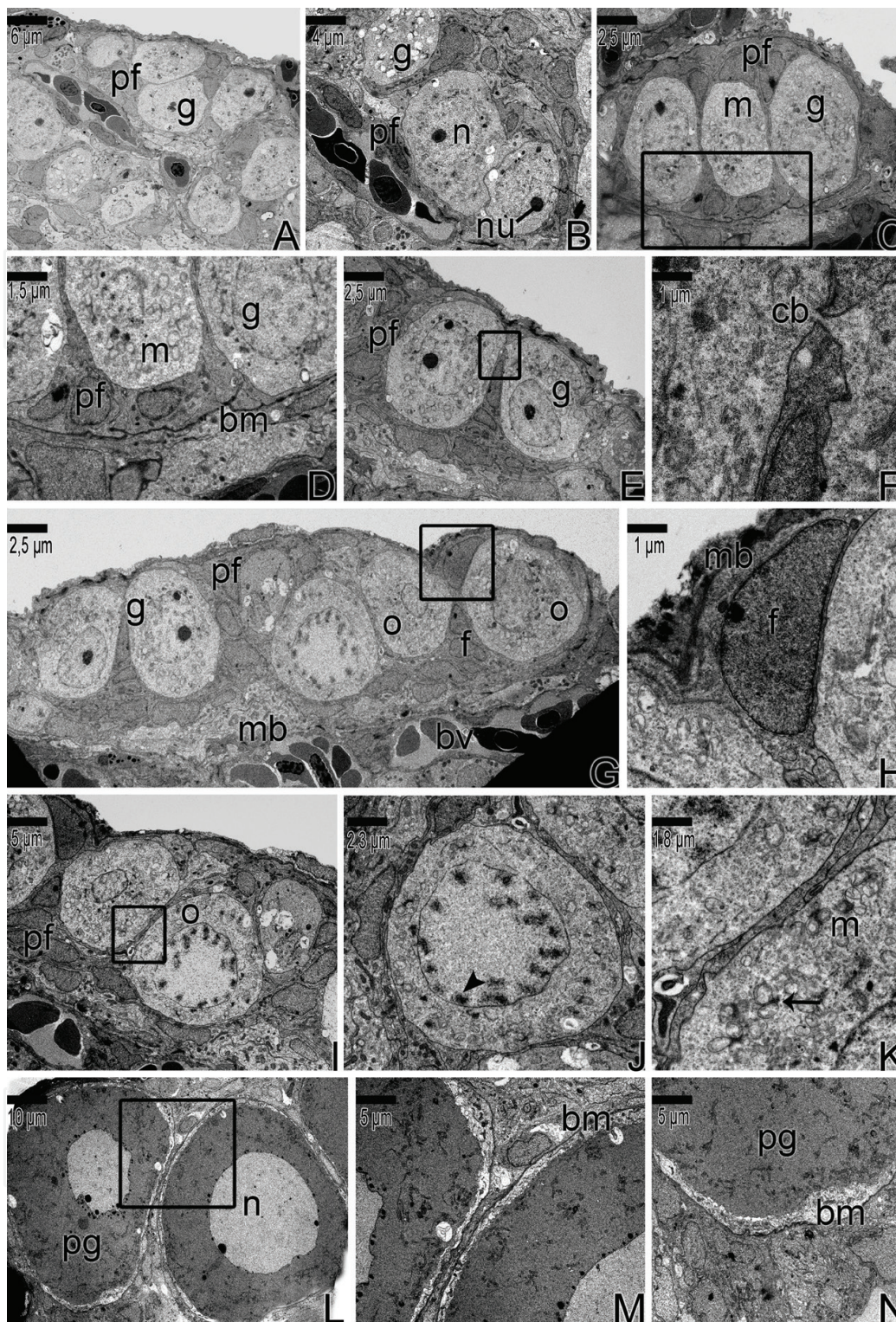


Figure 3. Transmission electron microscopy of *A. nigrofasciata* ovaries, showing details of the process of folliculogenesis. In the gonadal tissue, the oögonia (g) are encompassed by somatic cells, pre-follicle cells (pf), forming germline cysts (A–C), delimited by a basement membrane (bm) (C,D). In the germline cysts, the oögonia are interconnected by cytoplasmic bridges (cb) (E,F). The germline cysts of oögonia, oocytes (o), and isolated oögonia are immersed in the gonadal tissue, separated from the other somatic components by a basement membrane in formation (G–K). After folliculogenesis, the follicle complex is formed around a primary growth oocyte (pg) (L–N). Blood vessel (bv), nucleus (n), nucleolus (nu), mitochondria (m), follicle cell (f), synaptonemal complexes (arrowhead), and nüage (arrow).

primary growth, presents a nucleus with a variable number of nucleoli, which, initially located in the central region of the nucleus (oocytes with multiple nuclei), later become peripheral (perinucleolar oocytes).

In *T. albonubes*, the stage of the first folliculogenesis is quite rapid. Thus, with only 44 dpf and 1.5 cm long, the gonad is still thin, but it already presents diplotene oocytes in primary growth (Figure 4).

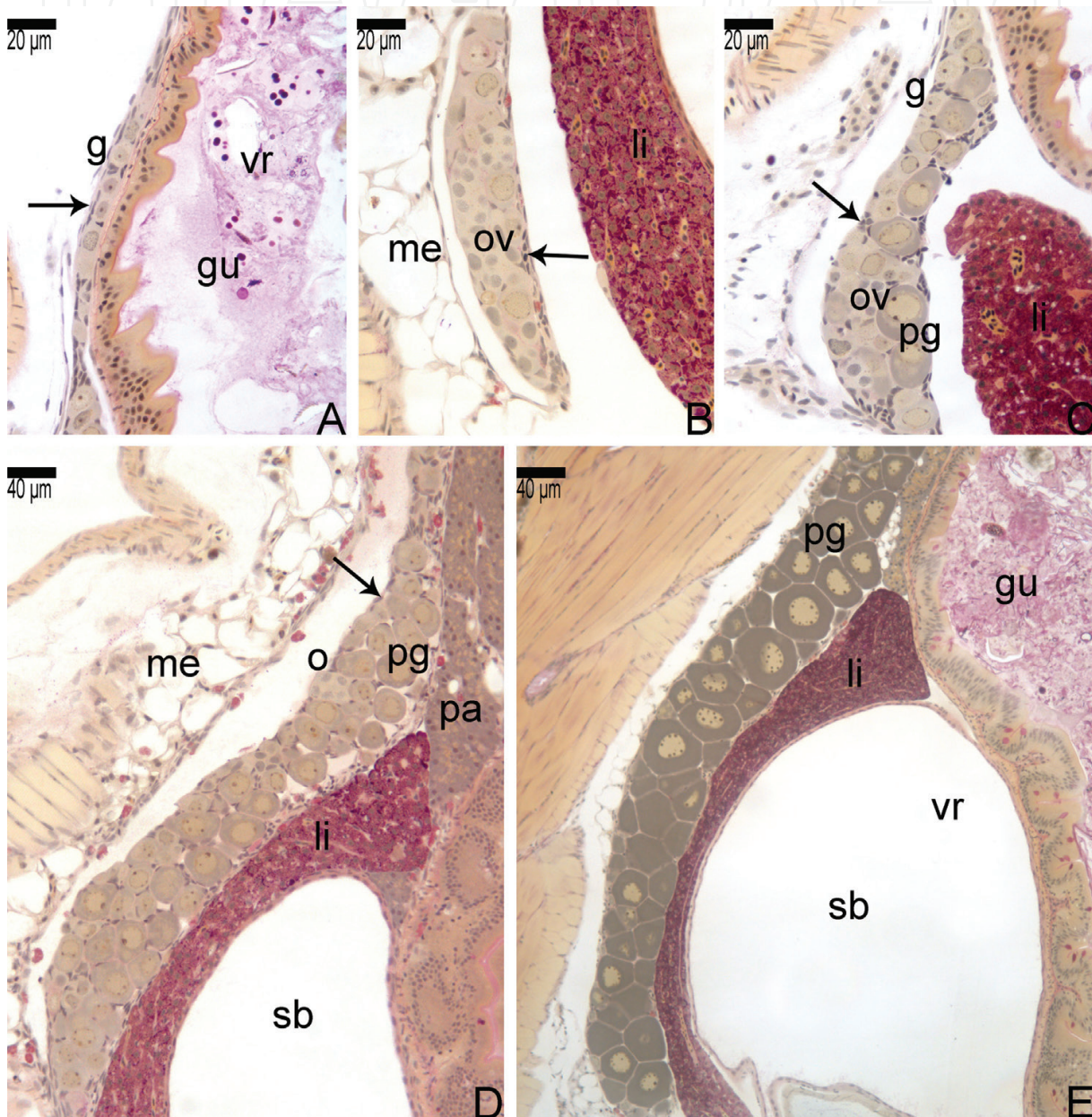


Figure 4. Parasagittal histological section of the female gonads in *T. albonubes* showing the development of the compact gonad, formed by oogonia (g) in A, and prophase oocytes (o) in different stages of the folliculogenesis (B and C). The diplotene oocytes enter into primary growth (D), becoming larger and basophilic (E). Ventral region (vr), pre-follicle cells (arrow), primary growth oocyte (pg), mesentery (me), liver (li), gut (gu), pancreas (pa), and swim bladder (sb). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

In *C. schwartzi*, this process appears to be slower. The gonad remains for a long period, from 130 to 150 dpf, presenting only germline cysts of oogonia and prophase oocytes. These cysts are separated from each other by a developing interstitial tissue, which responds positively to PAS and increases in number, gradually increasing the thickness and volume of the gonad (**Figure 5A–F**). At 160 dpf, in animals with 4 cm, the diplotene oocytes enter into primary growth, but the germline cysts are still predominant, and the gonad is still compact (**Figure 5G and H**).

In *A. nigrofasciata*, the folliculogenesis begins after 37 dpf. The gonadal tissue increases in length and thickness (**Figure 6A and B**). Oogonia decrease in quantity. Leptotene, zygotene, pachytene, and early diplotene oocytes become numerous and are easily identifiable (**Figures 6C–G and 7A**). The gonadal tissue presents a large amount of primary growth oocytes and remains with the same histological characteristics until the animal completes 90 dpf (**Figure 7B and C**), when presents 2 cm.

At this stage of ovarian differentiation, the gonad is still compact in all the species here analyzed (**Figures 4–7**).

2.5. Formation of the ovarian cavity

In most Teleostei fish, the ovaries are even saculiform organs, presenting a cavity in their interior. This type of ovarian organization is unique among vertebrates and is known as cystovarian ovary [32]. In this type, the ovaries are cavitory organs and present the germinal compartment in the form of lamellae, which protrude from the capsule toward the lumen of the organ. In this case, the ovarian cavity is continuous with the gonoducts [33], which merge caudally and flow into the urogenital papillae [3, 33].

The species utilized herein as representatives of Teleostei have this type of ovarian organization. The constitution of the ovary as a cavitory organ, and therefore the formation of the ovarian lumen, precedes the complete formation of the ovigerous lamellae in all of them, and, depending on the species, it may be concomitant to the constitution of the germinal epithelium. In all cases, the closure of the organ is gradual and can be followed through cross histological sections. Variations of the involved events can occur among the species studied.

In these species, the process of formation of the ovarian cavity follows what has been reported for most of fish [13] and is a result of the proliferation of somatic cells in the periphery of the ovary. This proliferation is responsible for the formation of the laminar tissues, which expand laterally and fuse, enclosing the forming ovary in a cavity—the before-known coelomatic cavity—now, the ovarian lumen.

Despite the similarities found during the cystovarian formation between the groups of fish analyzed, a single divergence could be observed, namely, the location of the somatic cell proliferation regions in the ovary and the direction (ventral or dorsal) of the laminar tissue toward the coelomic cavity.

In the Cypriniformes *T. albonubes*, the laminar tissues grow dorsally to the ovary (**Figure 8**). In contrast, in Perciformes *A. nigrofasciata*, the laminar tissues grow ventrally to the ovary

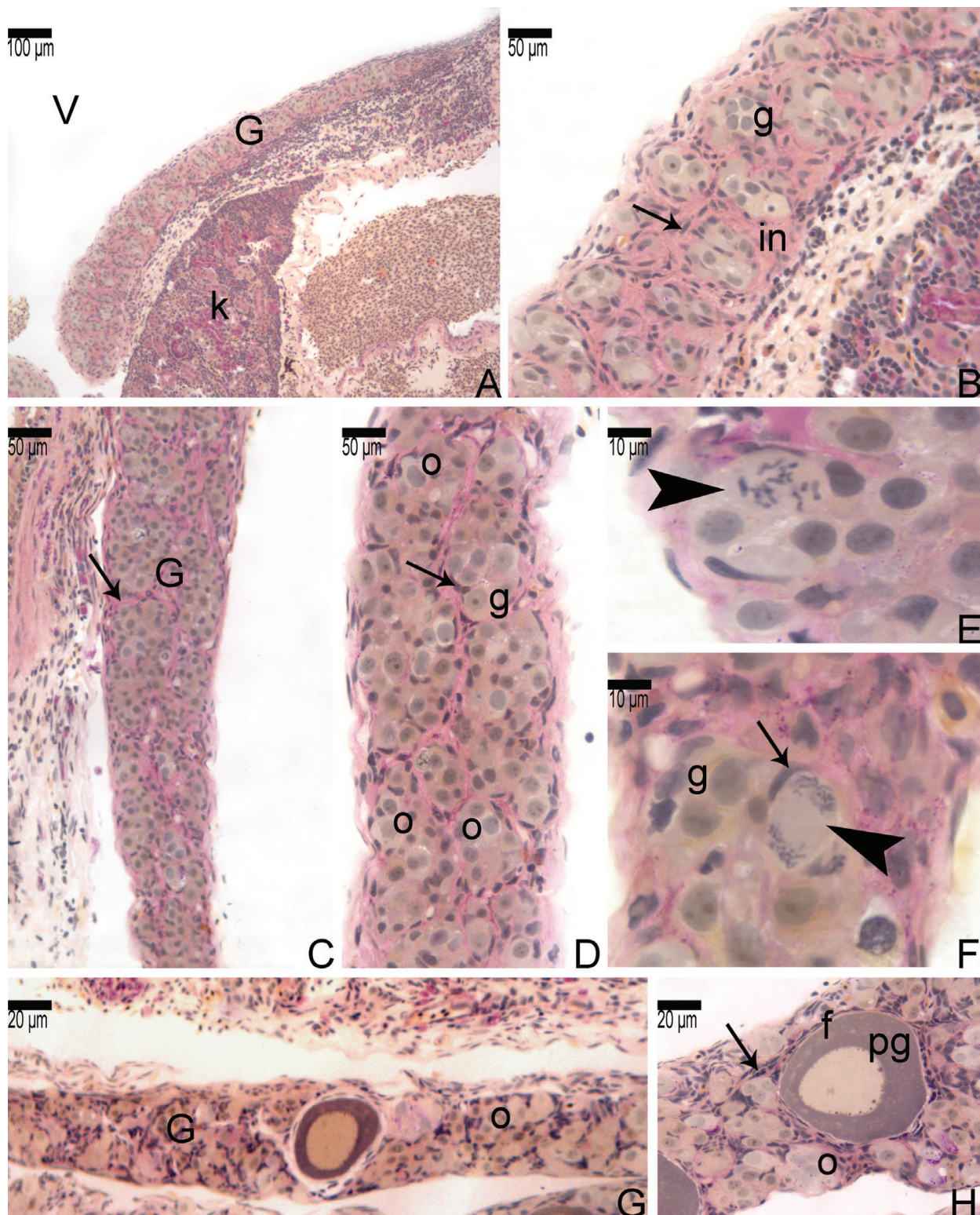


Figure 5. Parasagittal histological section of the female gonads in *C. schwartzi* showing the development of the compact gonad, formed by oogonia (g) and prophase oocytes (o) in different stages of the folliculogenesis. Note the developed interstitial tissue (in) (A–F) and the mitotic activity of oogonia (arrowhead) (E and F). Kidney (k), ventral region (v), pre-follicle cells (arrow), primary growth oocyte (pg), follicle cells (f), and gonad (G). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

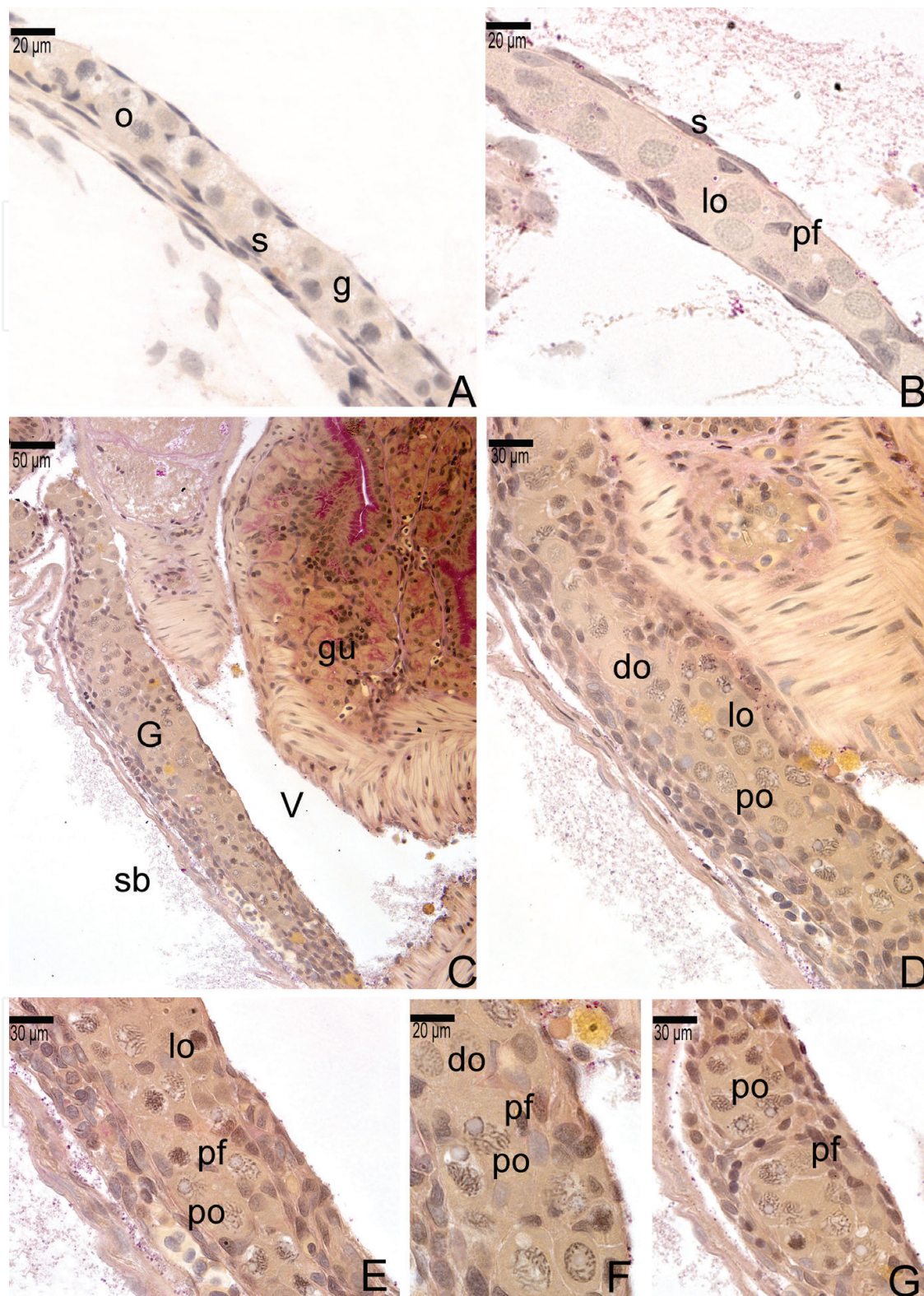


Figure 6. Parasagittal histological section of the female gonads in *A. nigrofasciata* showing the development of the compact gonad, formed by oogonia (g) and prophase oocytes (o) in different stages of the folliculogenesis. Ventral region (V), somatic cells (s), gut (gu), leptotene oocyte (lo), pachytene oocyte (po), diplotene oocyte (do), pre-follicle cells (pf), gonad (G), and swim bladder (sb). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

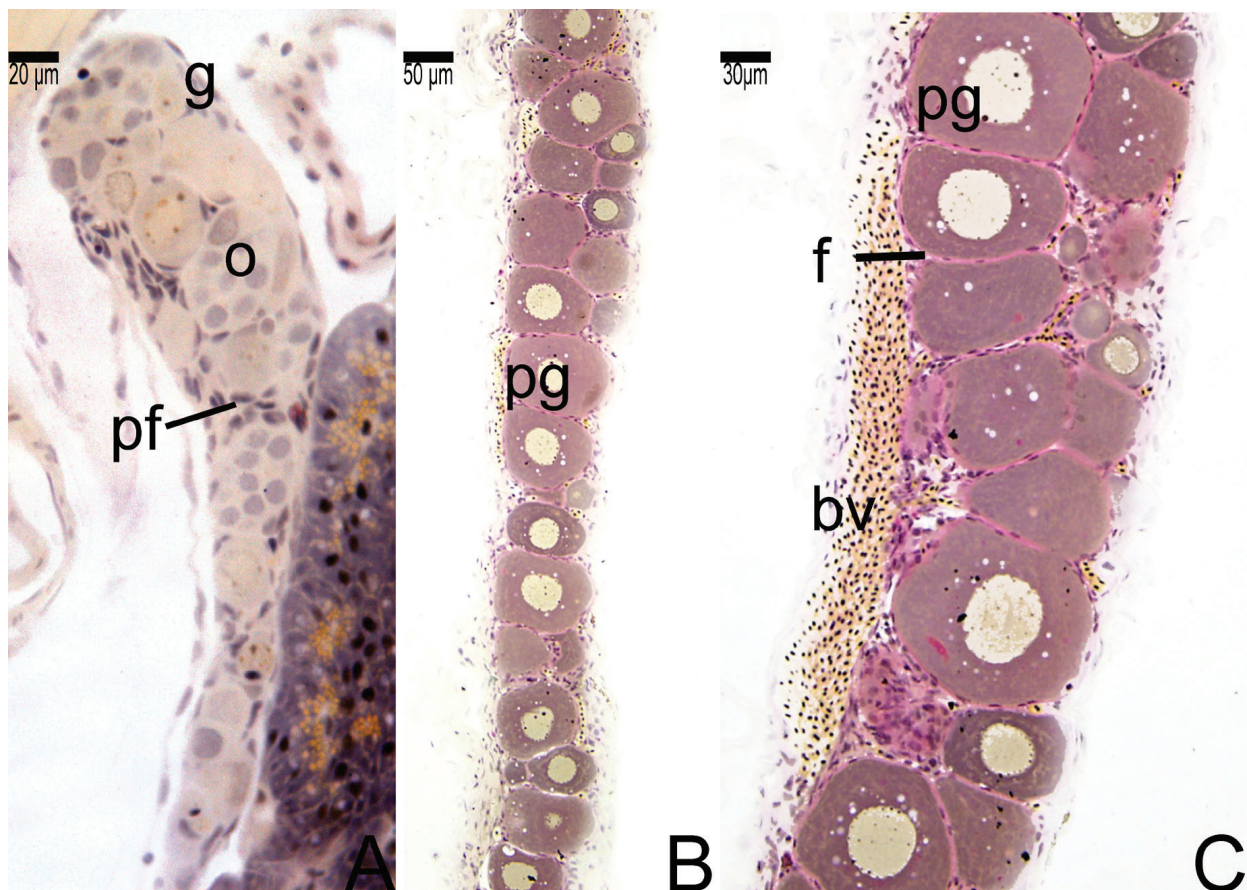


Figure 7. Parasagittal histological section of the female gonads in *A. nigrofasciata* in early development (A), showing prophase oocytes (o) and entrance of the oocyte in primary growth (pg) (B and C). Pre-follicle cells (pf), follicle cells (f), and blood vessel (bv). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

(**Figure 9**), similar to what occurs in *Cyprinus carpio*, another Cypriniformes [16], or in *G. ternetzi*, a Characiformes [21]. Therefore, the direction of the closure of the ovarian cavity seems to vary among species, independent on their phylogenetic position.

In *T. albonubes* and *A. nigrofasciata*, despite pertaining to different orders, being a basal and another derived taxa, respectively, the formation of the ovarian cavity (at 44 and 90 dpf, respectively) is very similar.

In both species, concomitant to the entrance of the oocyte in primary growth, the gonadal tissue, still compact, presents lateral tissue projections from the proliferation of somatic cells in the periphery of the gonadal tissue. Through the cross sections, the growth of the laminar tissue, on both sides of the ovary, can be traced toward the dorsal (*T. albonubes*) and ventral portion (*A. nigrofasciata*) of the gonad (**Figures 8 and 9**). During the growth of the laminar tissues, they eventually find the epithelium of the mesentery in the dorsal or ventral region, in which the gonad is supported. In this way, the laminar tissues fuse to the mesothelium and enclose the ovary in a space—the ovarian cavity. At this stage, oogonia and germline cysts, immersed in the still compact gonadal tissue, are concentrated in the periphery, near the newly formed

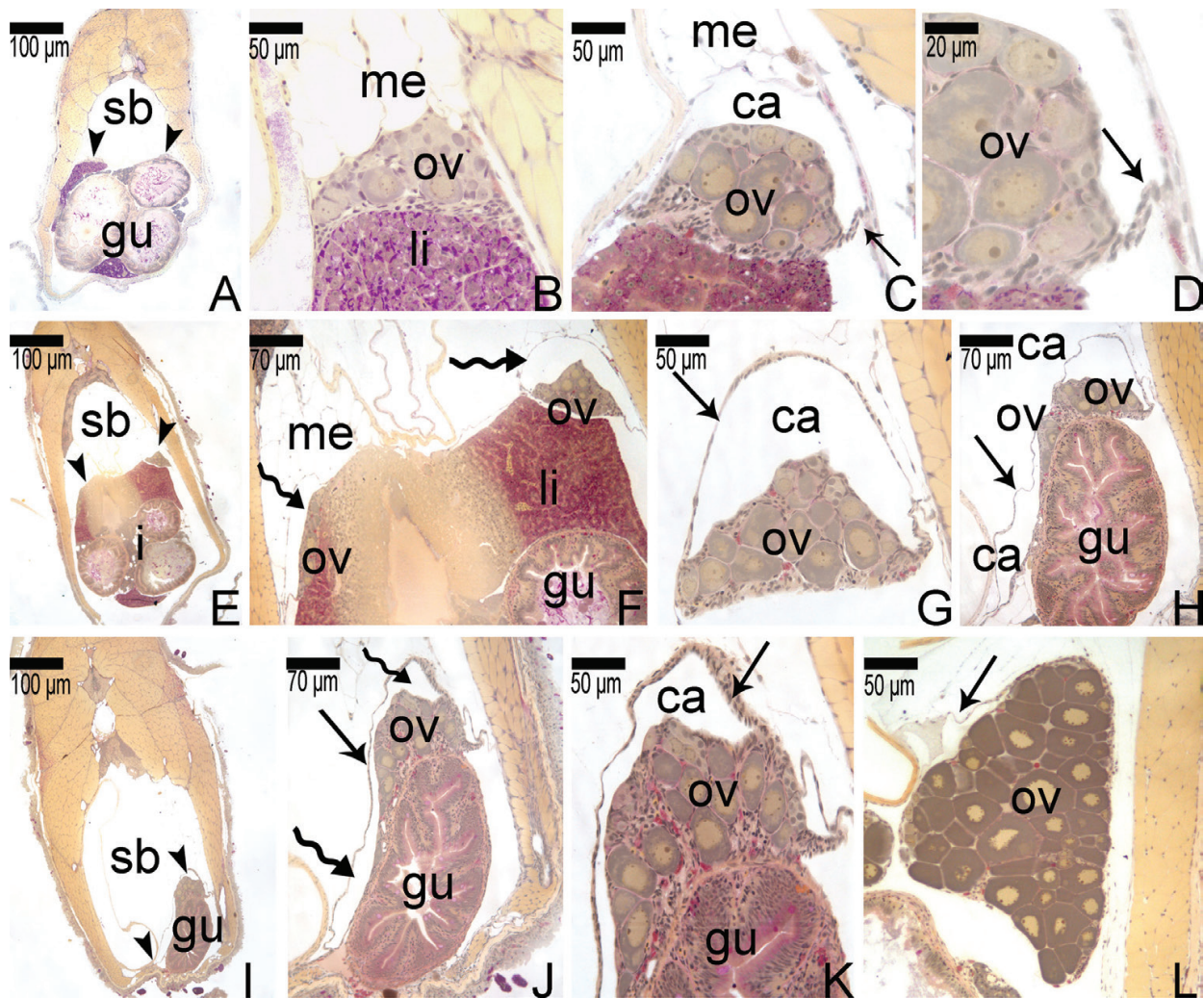


Figure 8. Histological cross sections of the female gonads in *T. albonubes*—formation of the ovarian cavity toward the dorsal region of the gonad. Localization of the ovaries in the coelomic cavity (A). Compact ovary (B) and ovary with lateral projections (C, D). Ovarian cavity (E–G). Note the ovarian cavity separated in each of the ovaries (E, H), forming a single cavity in the caudal region of the animal (I–K). Differentiated gonad with ovarian cavity in the dorsal region (L). Ovaries (arrowheads), mesentery (me), gut (gu), ovary (ov), ovarian cavity (ca), laminar tissue (arrow), swim bladder (sb), and ovarian lumen (sinuous arrow). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

ovarian cavity, whereas the primary growth oocytes occupy the opposite region. This is the first indication of the germinal epithelium formation in *T. albonubes* and *A. nigrofasciata*.

In *C. schwartzi*, another Ostariophysi (i.e., a basal taxa), the ovary is also considered a cystovarian, although the formation of the ovarian cavity occurs by a different mechanism, known as cavitation. In this, the formation of the ovarian cavity is the result of a reorganization of somatic components inside the gonadal tissue, during the formation of the ovigerous lamellae. Thus, in this species, the formation of the ovarian cavity is concomitant to the formation of the ovigerous lamellae and occurs at 180 dpf, in animals with 5 cm. The process will be described below.

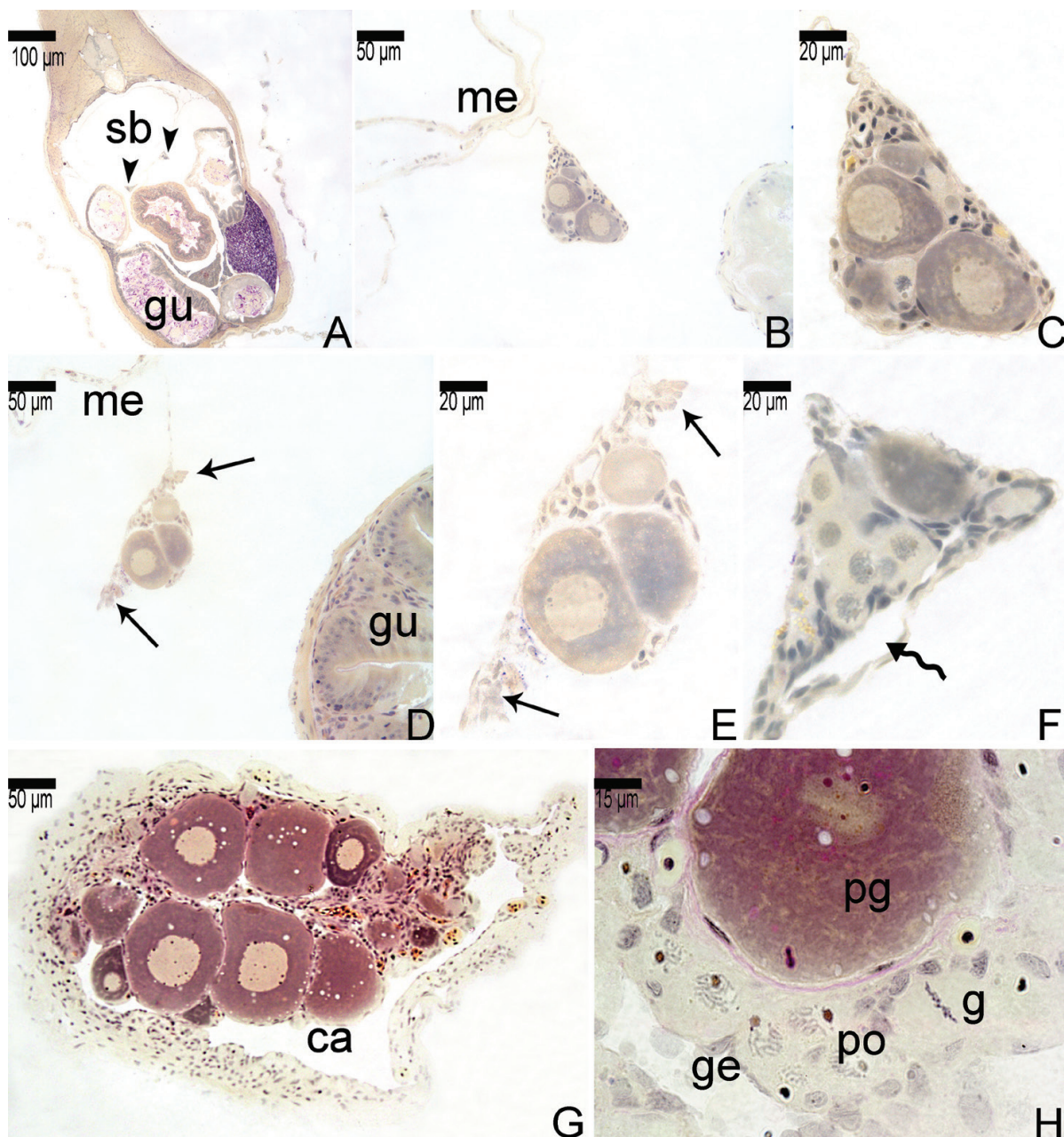


Figure 9. Histological cross section of the female gonads in *A. nigrofasciata*—formation of the ovarian cavity toward the ventral region of the gonad. Localization of the ovaries in the coelomic cavity (A). Ovary compact (B, C) and with lateral projections (D, E). Formation of the ovarian cavity (F) and the differentiated gonad (G), with established germinal epithelium (ge) (H). Ovaries (arrowheads), mesentery (me), gut (gu), ovarian cavity (ca), laminar tissue (arrow), swim bladder (sb), primary growth oocyte (pg), pachytene oocyte (po), and oogonia (g). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

2.6. From the formation of female germinal epithelium to the organization of follicle complexes

In all the species analyzed here, the gonadal tissue is still compact in the stage that precedes the formation of the ovigerous lamellae, even though the ovarian cavity is already formed. In the gonadal tissue, the developing ovarian follicles are gradually surrounded by a basement

membrane (**Figure 10**), remaining immersed in the gonadal tissue, along with germline cysts of oogonia, of prophase oocytes and isolated oogonia [16].

In *T. albonubes* with 60 dpf and in *A. nigrofasciata* with 100 dpf, both animals with 2 cm long, from this stage, epithelial cells in movement coming from the gonadal periphery invade the compact tissue forming invaginations that progress into the gonadal tissue, forming interlamellar spaces, which become deeper and deeper (**Figure 11**).

Thus, the lamellae gradually increase in size and project into the ovarian cavity. In the region of projection and formation of the ovigerous lamellae, the somatic cells peripheral to the gonadal tissue reorganize forming an epithelium that borders the newly formed lamellae. This newly formed epithelium isolates the germ cells from the interlamellar lumen. This mechanism, at the same time, forms the ovigerous lamellae and originates the germinal epithelium that borders the lamellae (**Figure 12**) [16].

In *C. schwartzi*, at 180 dpf (5 cm in length), the somatic components within the gonadal tissue undergo some reorganization, resulting in an alignment of the somatic cells throughout the longitudinal extent of the gonad. Thus, in longitudinal sections, there are several double rows parallel to each other and longitudinal to the gonadal tissue, from the cranial toward the caudal region of the gonad. These rows gradually move away from each other, giving rise to a small space that becomes more and more prominent (**Figure 13A–H**).

With the distancing of several longitudinal parallel rows of somatic cells to provide the ovarian lumen formation, the gonadal tissue is separated longitudinally in its central-medial region, becoming pleated. Thus, several parallel longitudinal pleats are formed, each one delimited by the somatic cells that originated them, composing the primordium of the ovigerous lamellae (**Figure 13I and J**).

Within each newly formed ovarian lamellae, the oogonia and germline cysts reorganize and migrate to the lamellar periphery, associating with the epithelial somatic cells that compose the border of each ovigerous lamellae, thus constituting the female germinal epithelium in *C. schwartzi*.

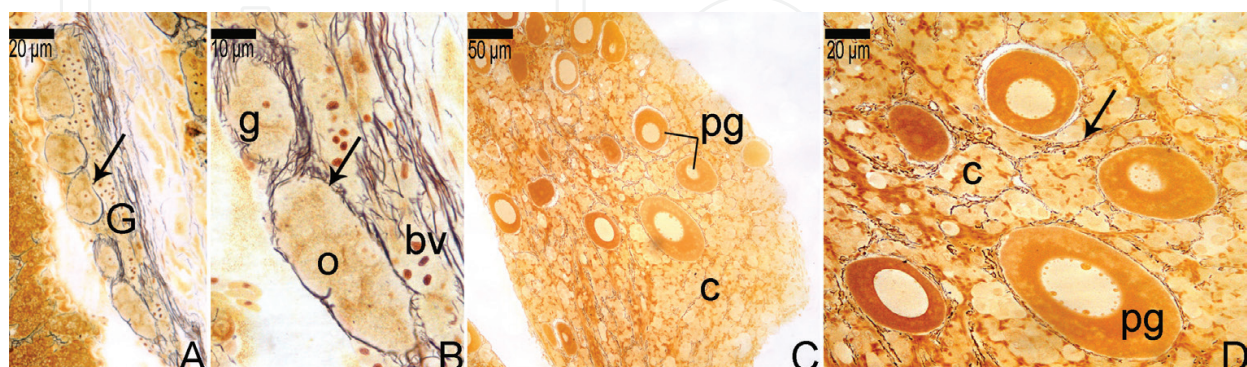


Figure 10. Histological section of the female gonads in *C. schwartzi*—Reticulin method. The germinal components are totally separated from the somatic components in the first stages of gonadal differentiation (A, B). (B) Detail of A. Note the cysts of oogonia (g) and cysts of prophase oocytes (o) surrounded by the basement membrane (arrow). (C and D) After entrance in primary growth (pg), each oocyte is individualized totally by the basement membrane (arrow). (D) Detail of C. Blood vessel (bv), germline cysts (c), and gonad (G).

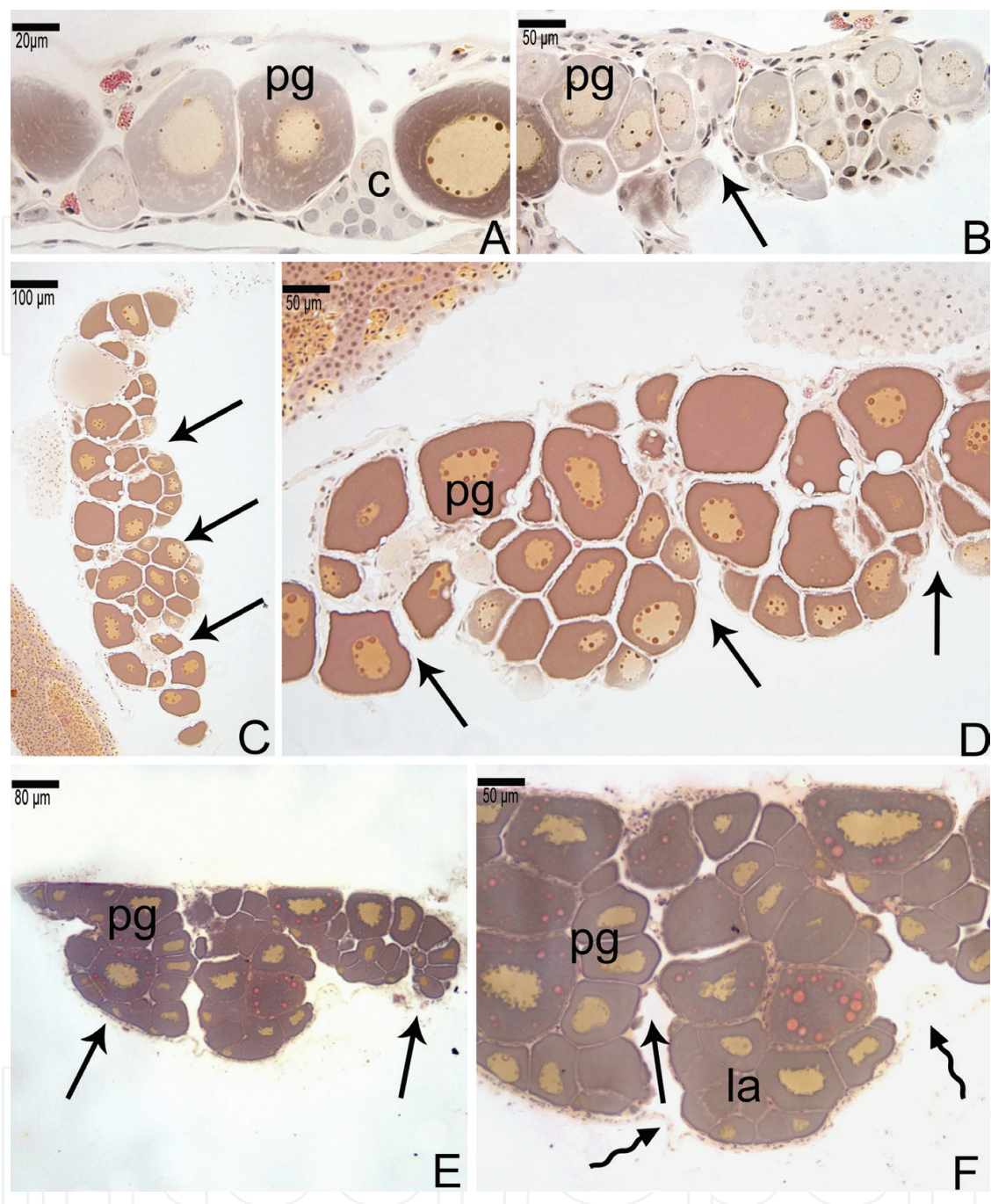


Figure 11. Parasagittal histological section of the female gonads in *T. albonubes*. Formation of the ovigerous lamellae (A–D) and establishment of the germinal epithelium. Ovarian structure already differentiated in cross section, showing ovigerous lamellae in the dorsal region (E and F). Formation of ovigerous lamellae (arrow), germline cysts (c), primary growth oocyte (pg), delimitation of the ovarian cavity (sinuous arrow), and ovigerous lamellae (la). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

By a mechanism opposite to the other Ostariophysi (the Cypriniformes *Cyprinus carpio*), where the ovigerous lamellae arise by the invagination of somatic cells in the gonadal tissue [16], in the Siluriformes *C. schurtzi*, those lamellae are formed by evagination and growth of the gonadal tissue toward the lumen of the ovarian cavity, which is already established. In cross sections, the gonadal tissue presents a little prominent ovigerous lamellae, on both sides

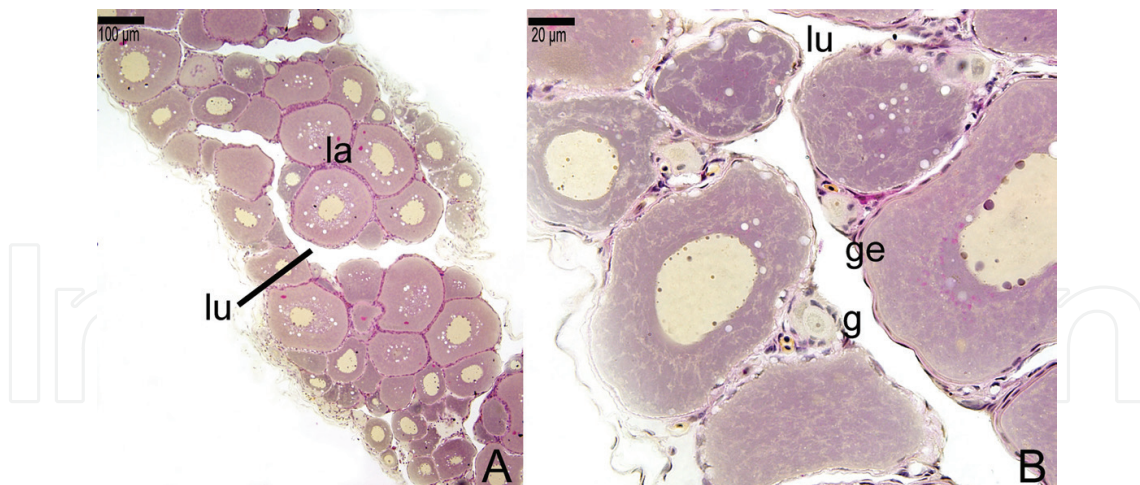


Figure 12. Parasagittal histological section of the female gonads in *A. nigrofasciata*. Ovigerous lamellae already formed (A) and establishment of the germinal epithelium (B). Ovarian lumen (lu), ovigerous lamellae (la), germinal epithelium (ge), and oogonium (g). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

of the ovary. With the advancement of the gonadal development, there is an expansion of the gonadal tissue toward the ovarian lumen, and the ovigerous lamellae become definite.

There are few reports on the different mechanisms that can lead to the formation of the ovigerous lamellae. Therefore, these mechanisms in other Teleostei are still quite unknown, making it impossible for an in-depth comparison along the evolutionary scale.

Thus, even though the formation of ovigerous lamellae is different in *C. schwartzi*, the process that follows for the establishment of the germinal epithelium is the same, even in species which do not present ovigerous lamellae, such as Poeciliids [24].

During the formation of the female germinal epithelium, the somatic epithelial cells, originated from specific regions according to each species, interpose among the germ cells, interconnecting them, after migrating through the compact gonadal tissue. The germ cells, from the beginning of the formation of the gonad, are segregated from other tissue components by the pre-follicle cells. These, in their turn, are supported on a basement membrane. Thus, the germinal epithelium, when formed, will be separated from the ovarian stroma by the basement membrane [16, 21].

In all the species analyzed here, along the female gonadal tissue, there are other cellular components which are interposed to the ovarian follicles already formed and to the cysts of oogonia and/or oocytes. Among these cellular components, small spaces arise and expand gradually giving rise to extravascular spaces. The extravascular spaces are filled by fluids rich in glycoproteins and polysaccharides. In adult animals, they originate from extravasation of blood plasma, which leaves the circulatory system between the endothelial cells and begin to fill regions within the gonadal tissue [15]. It is assumed that the extravascular spaces, in animals with gonadal differentiation, are formed in the same way [16].

Now, this fluid is disposed between the cellular components, moving them apart. Concomitantly, among the cellular components, star-shaped cells with mesenchymal characteristics interconnect progressively, forming a loose network that gives rise to an interstitial compartment [16].

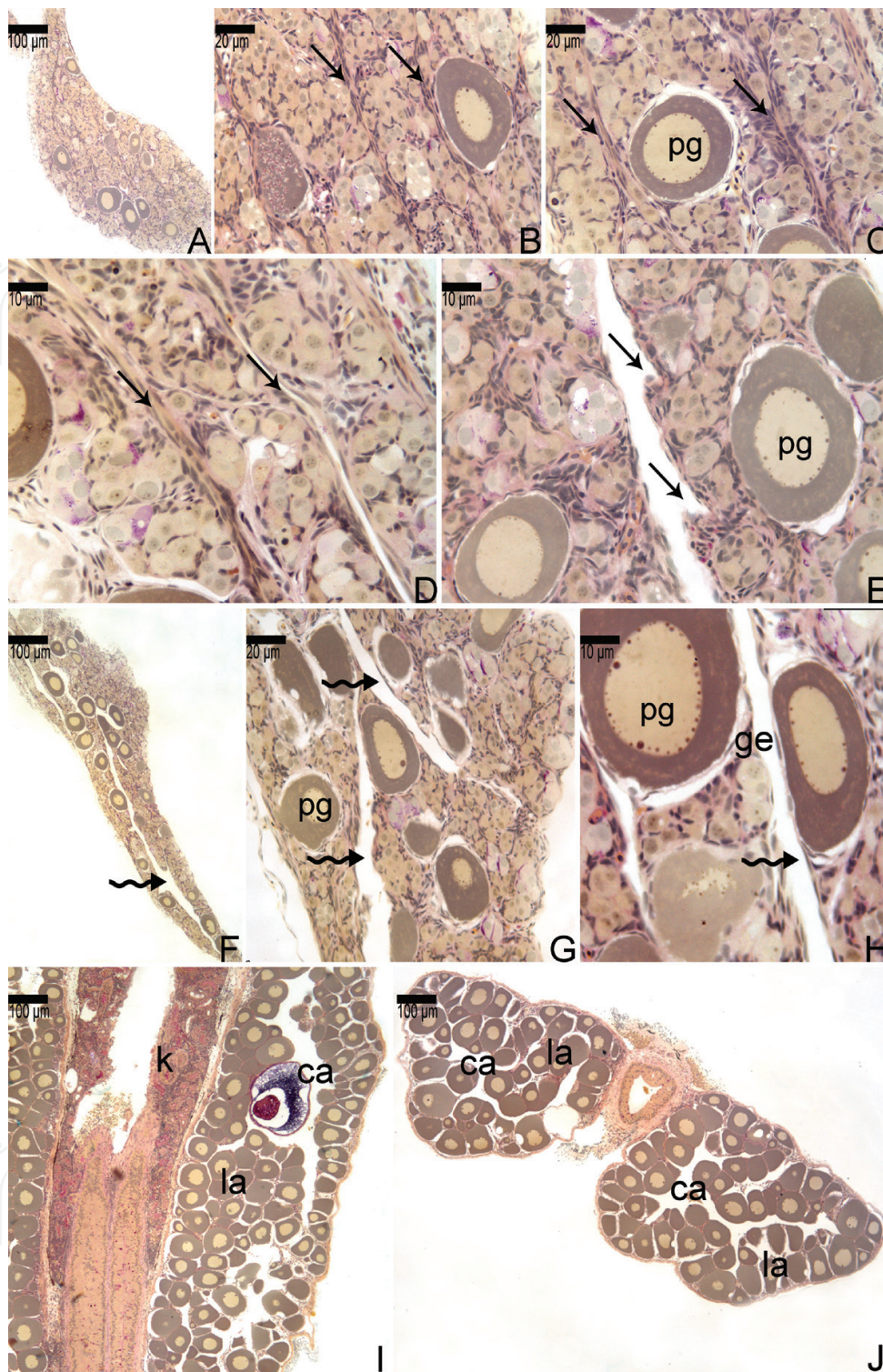


Figure 13. Parasagittal (A–H), longitudinal (I), and cross (J) histological section of female gonads in *C. schwartzi*. (A) Overview of the compact ovary. (B and C) Details of A, showing parallel rows of somatic cells (arrow) among germline cysts. (D and E) The parallel rows of somatic cells move away from each other, giving rise to spaces (arrow). (F) Overview of the ovary, showing spaces within gonadal tissue. (G and H) Note the spaces formed (sinuous arrow) and delimited the germinal epithelium (ge). The ovarian cavity and primordium of ovigerous lamellae are formed. (I and J) Ovary showing ovigerous lamellae (la) with defined ovarian cavity (ca) and established germinal epithelium. Kidney (k) and primary growth oocyte (pg). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

This compartment corresponds to the ovarian stroma, in which new cellular components will differentiate, remaining isolated from the germinal compartment by a basement membrane. The ovarian stroma in the fish is usually formed by a loose connective tissue, in which the extravascular spaces are larger and the amount of collagen fibers is small [16, 28].

From the newly formed stroma, the mesenchymal cells emit cytoplasmic projections which interact with the ovarian follicles and respond from now on by the formation of constituents of the theca. Since the follicles already have a totally formed basement membrane, the mesenchymal cells contacting the follicle, supported by their basement membrane, differentiate into a pre-theca cell and later into theca cells (Figure 14) [16].

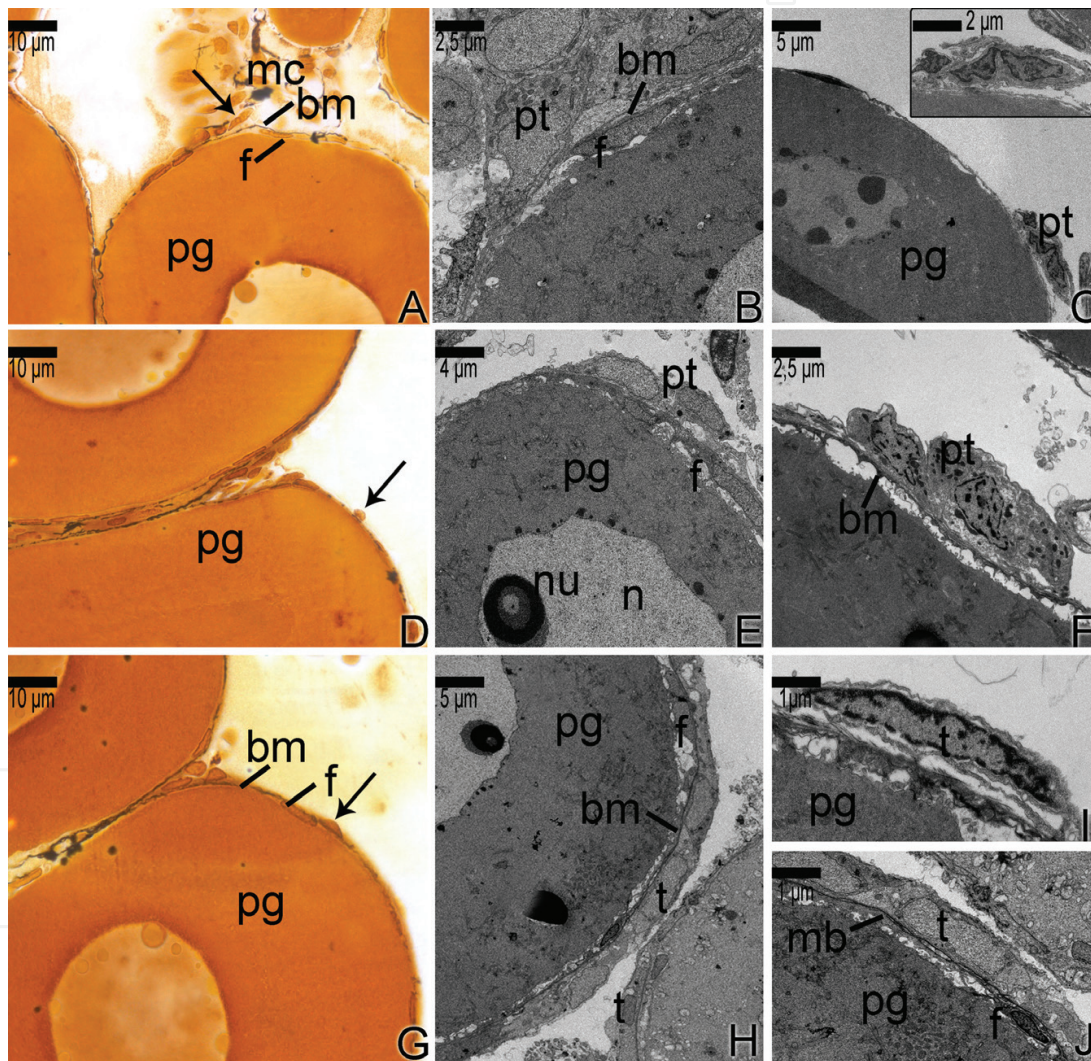


Figure 14. Histological section of the ovary in *C. schwanzi*—Reticulin method (A, D, G) and transmission electron microscopy of *A. nigrofasciata*. Formation of the theca from the mesenchymal cells (mc) of the ovarian stroma. (A) Ovarian stroma constituted by mesenchymal cells (mc), including pre-thecal cell (arrow). (B) Pre-thecal cell (pt) in the ovarian stroma. (C–F) The pre-thecal cell (arrow—pt) approaches the ovarian follicle, and it rests on its basement membrane (bm). (C—insert) Detail of the pre-thecal cell. (G–J) After this process, pre-thecal cell differs in theca (arrow—t) and changes its morphology becoming more fusiform. Primary growth oocyte (pg), follicle cell (f), nucleus (n), nucleolus (nu), and basement membrane (bm).

The ovarian stroma may be more or less developed, depending on the species. In *C. schwartzi*, it presents a developed stroma already in the early stages of gonadal differentiation. In contrast, *T. albonubes* and *A. nigrofasciata* initially exhibit a growth of gonadal tissue, and only in later stages of oocyte development, the gonad will present a developed stroma. Although some species such as *C. schwartzi* present developed interstitial components in the initial stages of the differentiation process, the ovarian stroma is only totally established later.

With the differentiation of the theca cells, the ovarian follicle becomes the follicle complex. The follicle complex is formed by the diplotene oocyte, surrounded by follicle cells, sustained by a basement membrane, and by two layers of theca cells [28, 33–36]. Thus, now the gonad presents two distinct compartments—the germinal epithelium of the ovigerous lamellae and the ovarian stroma [33, 35, 36]—separated by the basement membrane that becomes totally continuous (**Figure 15**).

Within the follicle complexes, the oocyte development proceeds. Microvilli arise in the oocyte plasma membrane and in the membrane of the apical region of the follicle cells. In this region, oocyte and follicle cells contact, and the formation of the zona pellucida begins [16, 29].

Once the germinal epithelium is fully established, it will become permanently active. In the epithelium, the oogonia proliferate forming clusters, denominated nests (**Figure 16A and B**). In these, the oogonia associate to the somatic cells of epithelial origin, differentiate, and form a new germline cyst (**Figure 16C–E**) [30]. Within the cyst, oogonia proliferate or enter into meiosis giving rise to germline cysts of prophase oocytes (**Figure 16F–K**). Isolated oogonia, oogonia inside cysts, cysts containing oocytes, and pre-follicle cells start occupying the inside of the same nest [16, 30].

After the formation of the ovarian follicle (**Figure 16L**), the oocyte follows its growth (**Figure 16M**), remaining connected to the germinal epithelium through a certain extension of the basement membrane shared between the follicle cells and the epithelial cells (**Figure 16N**) [16, 30, 33].

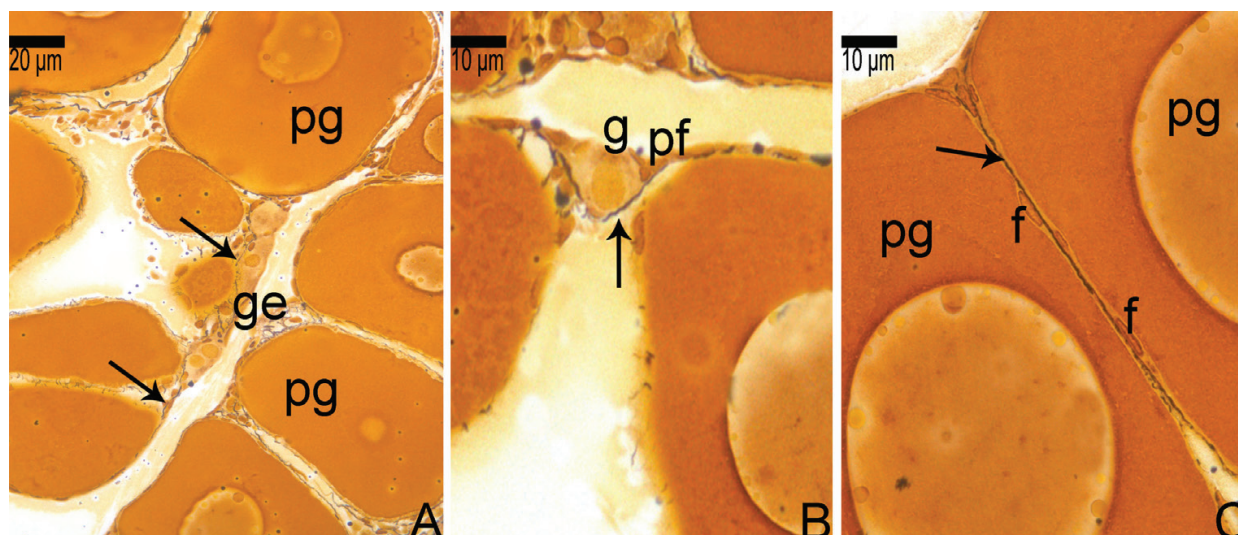


Figure 15. Histological section of the ovary in *C. schwartzi* — Reticulin method. The germinal epithelium is totally separated from the other somatic components (A). Detail of the germinal epithelium on basement membrane (B). Note the sharing of the basement membrane between two oocytes (C). Basement membrane (arrow), primary growth oocyte (pg), oogonia (g), cysts (c), germinal epithelium (ge), pre-follicle cells (pf), and follicle cells (f).

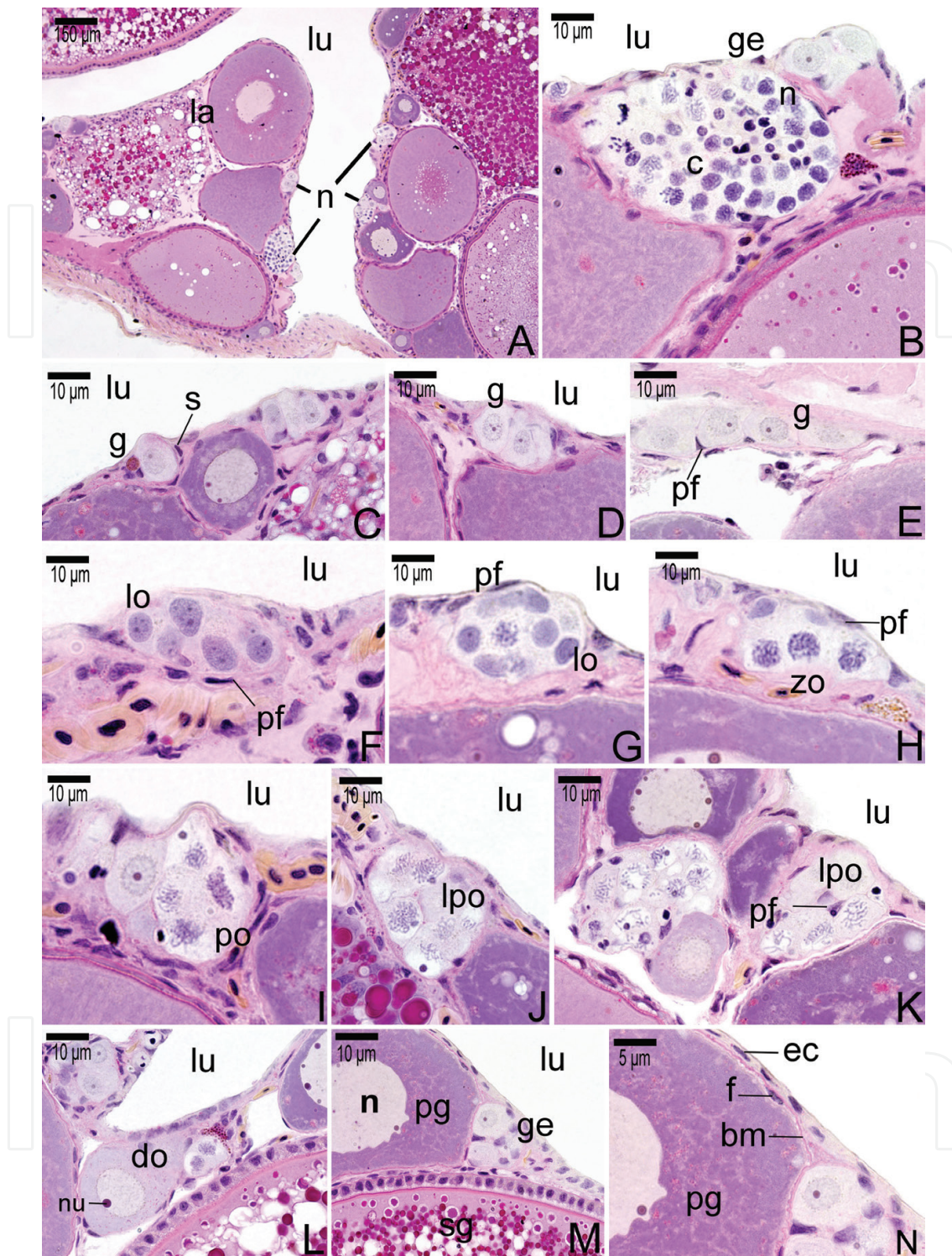


Figure 16. Folliculogenesis in a totally differentiated ovary of *A. nigrofasciata*. (A and B) Cell nests (n) in the germinal epithelium (ge). (C) Differentiated oogonia (g) isolated in the germinal epithelium. (D and E) Germline cysts of oogonia. (F and G) Cysts of leptotene oocytes (lo). (H) Cyst of zygotene oocytes (zo). (I and J) Cysts of pachytene oocytes (po). (K) Cyst of late pachytene oocytes (lpo) with pre-follicle cells (pf) invading the cyst and individualizing the oocytes. (L) Early diplotene oocyte (do) with one nucleolus (nu). (M) Primary growth oocyte (pg) connected to the germinal epithelium (ge). (N) Detail of (M), showing the region of sharing of the basement membrane (bm) between the oocyte and the germinal epithelium. Follicle cells (f), ovigerous lamellae (la), ovarian lumen (lu), germline cysts (c), nucleus (n), somatic cells (s), secondary growth oocyte (sg), and epithelial cell (ec). Staining: periodic acid Schiff + hematoxylin + metanil yellow.

Once the ovarian follicle is formed, i.e., the folliculogenesis process is complete, the oocyte effectively initiates its primary growth [36–38]. From here, the oocytes will be ready to respond to the stimuli that lead to the incorporation of the yolk, and therefore they undergo maturation and subsequent ovulation or spawning [35–38].

The species *T. albonubes*, *A. nigrofasciata*, and *C. schwartzi* analyzed here presented sexual maturity, and they were able to spawn after 180, 150, and 540 days postfertilization.

3. Conclusion

When analyzing different representatives of Teleostei, it can conclude that the processes involved in female gonadal differentiation are quite similar and it is possible to differentiate supposedly female and male gonads, even in the early development stages, independent on being a basal or derived species.

In the three species analyzed here, representatives of basal and derived taxa in Teleostei, the beginning of the female gonadal differentiation is marked by the entrance of the oogonia into meiosis, in early stages of the gonadal development, when the gonad is still a compact tissue. Thus, the formation of the ovarian cavity occurs only after the entrance into meiosis of the oocytes, preceding the formation of ovigerous lamellae in *T. albonubes* and *A. nigrofasciata*. In *C. schwartzi*, the formation of the cystovarian and the establishment of the ovigerous lamellae occur simultaneously. Despite the differences, the folliculogenesis is a very conserved process among basal and derived taxa, with no difference between species.

Thus, although there are differences in the chronology of the differentiation among species of Teleostei, the processes involved are quite similar and culminate in the formation of analogous structures in the different fishes. Therefore, these data showed here can be applied to the most different groups of Teleostei fish.

4. Complementary material

Methodology used: Larvae and juveniles were obtained from spawns of adult of the three species. After hatching, part of the brood was sampled periodically covering the period of histologically discernible sex differentiation. The specimens were anesthetized with 0.1% benzocaine and killed according to the institutional animal care protocols and approval (175/2009-CEEA-IBB/UNESP). The gonadal tissues were fixed by immersion in 2% glutaraldehyde and 4% paraformaldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.2) for at least 24 h.

For light microscopy, the gonadal tissue from larvae and juveniles was embedded in historesin (Leica HistoResin). Serial sections (3µm) were stained with periodic acid Schiff (PAS) + hematoxylin + metanil yellow [39] and with the reticulin method that enhances the basement membranes. Gonadal tissues were evaluated by using a computerized image analyzer (Leica

Qwin 2.5). The reticulin stain [40] uses an oxidizing agent, potassium permanganate, to oxidize aldehyde groups. Subsequently, the oxidized aldehyde groups are detected by the deposition of positive silver ions followed by their reduction using formalin. The result is a black hue of the reticulin fibers. As reticulin fibers are part of basement membranes, the method clearly detects basement membranes.

For electron microscopy, the gonadal tissue from larvae and juveniles was postfixed for 2 h in the dark in 1% osmium tetroxide (in the same buffer). To highlight the cellular structures, block-staining was carried out using an aqueous solution of 5% uranyl acetate for 2 h. Subsequently, the specimens were dehydrated and embedded in Araldite, sectioned, and post-stained with a saturated solution of uranyl acetate in 50% ethanol and 0.2% lead citrate in NaOH (1 N). Electron micrographs were obtained using a Tecnai Spirit Fei Company Transmission Electron Microscope.

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