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Development of the germinal epithelium and early folliculogenesis during ovarian morphogenesis in the cichlid fish *Cichlasoma dimerus* (Teleostei, Perciformes)

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Abstract

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Formation of the germinal epithelium and folliculogenesis during ovarian development in *Cichlasoma dimerus* were described at the light- and electron-microscopic levels. Prior to gonadal differentiation, germ cells and enveloping support cells reside within an inpocketing of the coelomic epithelium. Separation of the germinal and interstitial compartments of the gonad by a basement membrane is apparent from early gonadal development. Upon ovarian differentiation, oogonia undergo cyst-forming divisions leading to the formation of clusters of interconnected cystocytes that synchronously enter meiosis, becoming oocytes. At the pachytene step, each oocyte becomes individualized by cytoplasmic extensions of prefollicle cells, thereby developing as an ovarian follicle. Subsequent somatic reorganization leads to the formation of the ovarian lumen in a cephalocaudal gradient. As a result, the germinal epithelium becomes internalized and lines the ovarian lumen. As defined by its origin from the germinal epithelium, the ovarian follicle is composed of an oocyte and the surrounding follicle cells. Thecal cells derived from the stroma encompass the basement membrane outside the follicle, thus forming a follicle complex. A common basement membrane is shared by the germinal epithelium and the follicle complex along a small portion of its surface. This point of attachment represents the site at which the oocyte would be released to the ovarian lumen during ovulation.

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Introduction

In adult teleost fishes, folliculogenesis begins in the germinal epithelium that forms the surface of the ovigerous lamellae in the ovary. Within the germinal epithelium, germ cell renewal assures and maintains the continuity of oogenesis. A better understanding of folliculogenesis and oogenesis in fishes has been possible due to recent reports on the role of the germinal epithelium in ovarian follicle formation and on the functional events that regulate oocyte development (Grier 2000, 2012; Parenti and Grier 2004; Grier *et al.* 2005, 2007, 2009). However, most of the available data regarding the germinal epithelium come from studies on adult fishes. When or how the germinal epithelium is formed during ovarian morphogenesis in Teleostei is still unclear and a matter of debate, as it has been addressed only in one species, the common carp, *Cyprinus carpio* (see Mazzoni *et al.* 2010).

In teleosts, primordial germ cells arise at extragonadal locations and migrate to the genital ridges (gonadal anlagen) (Braat *et al.* 1999) where they become associated with

somatic cells. Although some studies on early gonadogenesis in teleosts are available, in most of them, it is not clear what types of somatic cells are present in the gonadal primordium and whether they belong to different tissue compartments. Ultrastructural analysis has shown that the source of somatic cells that surround the germ cells in the ovary of adult fish is the germinal epithelium that lines the ovarian lamellae and borders the ovarian lumen, as reported in the common snook, *Centropomus undecimalis* (see Grier 2000, 2012) and the rainbow trout, *Oncorhynchus mykiss* (see Grier *et al.* 2007). A basement membrane always separates the stroma from the germinal epithelium and its derivatives, the ovarian follicles. Follicle cells are reported to derive from epithelial cells (Wallace and Selman 1990; Selman *et al.* 1991; Grier 2000, 2012; Quagio-Grassiotto and Guimaraes 2003; Ravaglia and Maggese 2003; Grier *et al.* 2007; Quagio-Grassiotto *et al.* 2011) and to synthesize the basement membrane that surrounds the ovarian follicles (Le Menn *et al.* 2007; Lubzens *et al.* 2010). Coincidently, follicle cells have an epithelial origin in amphibians (Merchant-Larios and Villalpando 1981; Iwasawa and Yamaguchi 1984; Tanimura and Iwasawa 1988) and mammals (Sawyer *et al.* 2002; Mork *et al.* 2012). During teleost folliculogenesis, thecal cells become associated with the follicle, forming a follicle complex (Grier 2000). Thecal cells are reported to derive from the stromal mesenchyme subjacent to the germinal epithelium (Nagahama 1983; Begovac and Wallace 1988; Wallace and Selman 1990; Grier 2000; Quagio-Grassiotto and Guimaraes 2003; Ravaglia and Maggese 2003; Meijide *et al.* 2005; Grier *et al.* 2007; Mazzoni *et al.* 2010; Quagio-Grassiotto *et al.* 2011), coincidently with studies in mammals reporting that these cells originate from fibroblast-like precursor cells within the ovarian stroma (Young and McNeilly 2010). Here, we show that separation of the germinal epithelium and the stroma by the basement membrane is readily apparent from initial stages of gonad development, that is upon arrival of germ cells to the genital ridge.

A little known concept regarding ovarian gametogenesis in fish is the formation of germline cysts as intermediate structures in the developmental pathway from oogonia to ovarian follicles. A germline cyst is composed of germ cells that remain interconnected by intercellular bridges as a result of incomplete cytokinesis. The germline cyst is surrounded by peripheral somatic prefollicle cells, and during vertebrate folliculogenesis, it yields multiple oocytes in individual follicles (Pepling 2006; Marlow 2010). The formation of germline cysts is a conserved feature, as it has been reported to contribute to female gametogenesis in diverse animal species including both invertebrates, such as in insects (de Cuevas *et al.* 1997; Pepling *et al.* 1999), and vertebrates, such as in frogs and mammals (Pepling *et al.* 1999; Matova and Cooley 2001; Kloc *et al.* 2004; Pepling 2006). More recently, cysts have been also reported to occur in teleosts (Saito *et al.* 2007; Mazzoni *et al.* 2010; Quagio-Grassiotto *et al.* 2011; Wildner *et al.* 2013).

Ultrastructure of the ovaries of the syngnathid fishes, the gulf pipefish, *Syngnathus scovelli*, and the lined seahorse, *Hippocampus erectus*, was described by Wallace and Selman (1990) and Selman *et al.* (1991), respectively. In these species, follicles are produced by a germinal ridge which contains oogonia, early meiotic oocytes and prefollicle cells. The germinal ridge was described by these authors as an 'outpocketing' of the luminal epithelium, as indicated by a continuous underlying basement membrane. During folliculogenesis, prefollicle cells invest diplotene oocytes and the follicle thus formed eventually pinches off from the germinal ridge as a primordial follicle surrounded by a basement membrane derived from the germinal ridge. This description of the syngnathid germinal ridge provided insights into a possible way the ovarian germinal epithelium might develop in teleosts, as we propose here.

The acará, *Cichlasoma dimerus*, is a freshwater cichlid fish from South America (Nelson 2006) that adapts easily to captivity and shows notable reproductive features such as a relatively high spawning frequency (about every 30 days during 8 months of the year, under laboratory conditions) and acceptable survival rates, providing an appropriate model for developmental studies. In a previous study (Meijide *et al.* 2005), we analysed the pattern of gonadal sex differentiation of this species and established that *C. dimerus* is a differentiated gonochorist in which ovaries and testes develop directly from undifferentiated gonads. Here, we describe the development of the germinal epithelium and the process of folliculogenesis during early ovarian morphogenesis, with special emphasis on the spatial relations between different cell types and the basement membrane. In particular, we provide an ultrastructural evidence-based interpretation of how the germinal epithelium is formed upon arrival of germ cells to the genital ridge and later develops as the site from where ovarian follicles are originated.

Materials and Methods

Adult *Cichlasoma dimerus* used in this study were captured in Esteros del Riachuelo, Corrientes, Argentina (27° 25′S, 58° 15′W) by local fishermen and transferred to the laboratory. Single pairs were kept in 45 L aquaria, at 26.5 ± 1 °C and a 12:12 h photoperiod. Laboratory aquaria were well aerated and provided with a layer of gravel and smooth stones for egg deposition on the bottom. Fish were fed with pelleted commercial food, supplemented with *Tubifex* worms.

Eggs and larvae were obtained from several natural spawns of six pairs. On the 14th day after spawning (12 days posthatching), each lot of offspring was isolated in a 20 L aquarium, where their development was followed until the juvenile stage was reached. A part of the brood was sampled periodically from day 14 to day 100 postfertilization (6 ± 0.5 mm–22 ± 3 mm TL), covering the period of histologically discernible sex differentiation. Temperature and photoperiod conditions were the same as those of the adults. Fry

were initially fed with freshly hatched nauplii of *Artemia salina* and later fed finely ground, dried flake food.

Specimens were anesthetized with 0.1% benzocaine and killed according to the guidelines on the care and use of fish in research and testing from the Canadian Council on Animal Care (2005). Gonads of larvae and juveniles were obtained from fish immediately after sacrifice and processed according to standard techniques for light and electron microscopy. In the case of undifferentiated and recently differentiated fish (14–65 days postfertilization), the median region of the body was fixed; while just the dissected ovaries were processed in the case of differentiated females (66–100 days postfertilization). For light microscopy, samples were fixed in Bouin's liquid, gradually dehydrated and embedded in paraffin. Sections, 7 μm thick, were stained with haematoxylin–eosin. The slides were examined and photographed with a Nikon Microphot FX. For transmission electron microscopy (TEM), samples were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4–8 h. The tissue was then rinsed in 0.1 M phosphate buffer and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h. Tissue was subsequently rinsed in distilled water, dehydrated in a graded alcohol series and acetone, and embedded in Spurr resin. Ultrathin sections were made with a Sorvall MT2-B ultramicrotome, stained with aqueous uranyl acetate and lead citrate, and examined under a Philips EM 301. Semithin sections, stained with toluidine blue, were used for orientation and photographed. Voucher slides were deposited in the Histological Collection at the Departamento de Biodiversidad y Biología Experimental, FCEN, Universidad de Buenos Aires under numbers 020598-110. Oocyte development and folliculogenesis were described in accordance with Grier *et al.* (2009).

Results

The germinal epithelium in the undifferentiated gonad

Between days 14 and 38 postfertilization, paired undifferentiated gonadal primordia are observed within the abdominal cavity, suspended from the dorsal peritoneal lining by short mesenteries, at both sides of the dorsal gut mesentery (Fig. 1A). Cross sections reveal that the gonadal primordium consists of large germ cells surrounded by enveloping somatic cells (Fig. 1 A–D,H). Sexually undifferentiated germ cells contain round, euchromatic nuclei with one or two prominent nucleoli and a cytoplasm rich in membranous organelles (Fig. 1A,G,H). Somatic cells possess heterochromatic nuclei of various shapes and relatively little cytoplasm containing few organelles. Although somatic cells look similar to one another, two types of cells can be distinguished according to their position relative to the basement membrane, that is according to the tissue compartment in which they occur. The first type is represented by the coelomic epithelial cells (mesothelial cells) and the derived support cells. Epithelial cells are located at the periphery of the gonadal anlage, while support cells occur in an interior position, where they become associated with germ cells (Fig. 1B–D). Epithelial cells are connected to each other by desmosomes and tight junctions. They possess an elongated nucleus and show picnocytotic vesicles within the cytoplasm (Fig. 1B–F). Support cells tend to encompass germ cells with cytoplasmic processes (Fig. 1B–D,H) so that only in rare cases do germ cells directly contact the basement membrane (Fig. 1C). Before gonadal differentiation, mitotic figures indicative of germ cell proliferating activity are occasionally observed (Fig. 1B,C). During mitosis, germ cells may undergo kariokinesis without cytokinesis; as a result, no cell limits are initially observed between adjacent nuclei. Rather, a continuous cytoplasm containing organelles such as mitochondria and smooth endoplasmic reticulum occurs between them (Fig. 1G). Separation of the cytoplasm is subsequently facilitated by the growth of cytoplasmic processes of support cells between germ cell nuclei (Fig. 1G,H). Epithelial cells along with support and germ cells occur within the germinal compartment. Between epithelial and support cells, a thin space corresponding to the interstitial compartment of the developing gonad becomes evident. Initially, this space contains collagen fibrils and few somatic cells of the second type, namely interstitial mesenchymal cells, which are restricted to the dorsal region of the gonad (Fig. 1C–E,G). The gonadal interstitium is delimited 'at both sides' by a thin basement membrane (Fig. 1C–E). These 'two basement membranes' correspond to a folding of a single basement membrane, that is the basement membrane subjacent to the coelomic epithelium, and can occasionally fuse so that they appear as an only thick membrane (Fig. 1F). Within the gonad, the basement membrane separates two different tissue compartments, namely the germinal and the interstitial compartments.

The germinal epithelium during ovarian differentiation

Ovarian differentiation takes place from day 38 onwards in about half of the specimens, the other half corresponding to presumptive males, whose gonads remain undifferentiated. Within the differentiating ovary, germ cells, namely oogonia, undergo intensive mitotic proliferation and a subsequent entrance into meiosis, becoming oocytes (Fig. 2A–C). In addition, blood capillaries are observed in the dorsal region of the gonad for the first time (Fig. 2C). Oogonia contain a prominent rounded euchromatic nucleus with a large nucleolus, mitochondria with lamellar cristae, conspicuous nuage and abundant smooth endoplasmic reticulum. Generally, they appear as individual cells, surrounded by supporting prefollicle cells (Fig. 3A). Oogonial mitotic divisions yield a cyst of interconnected germ cells or cystocytes that enter meiosis and develop synchronously. As the different steps of meiotic prophase progress, the early oocytes display nuclei with distinctive patterns of chromatin organization. Thick strands of condensed chromatin become evident as formation of the synaptonemal complexes and pairing of the homologous

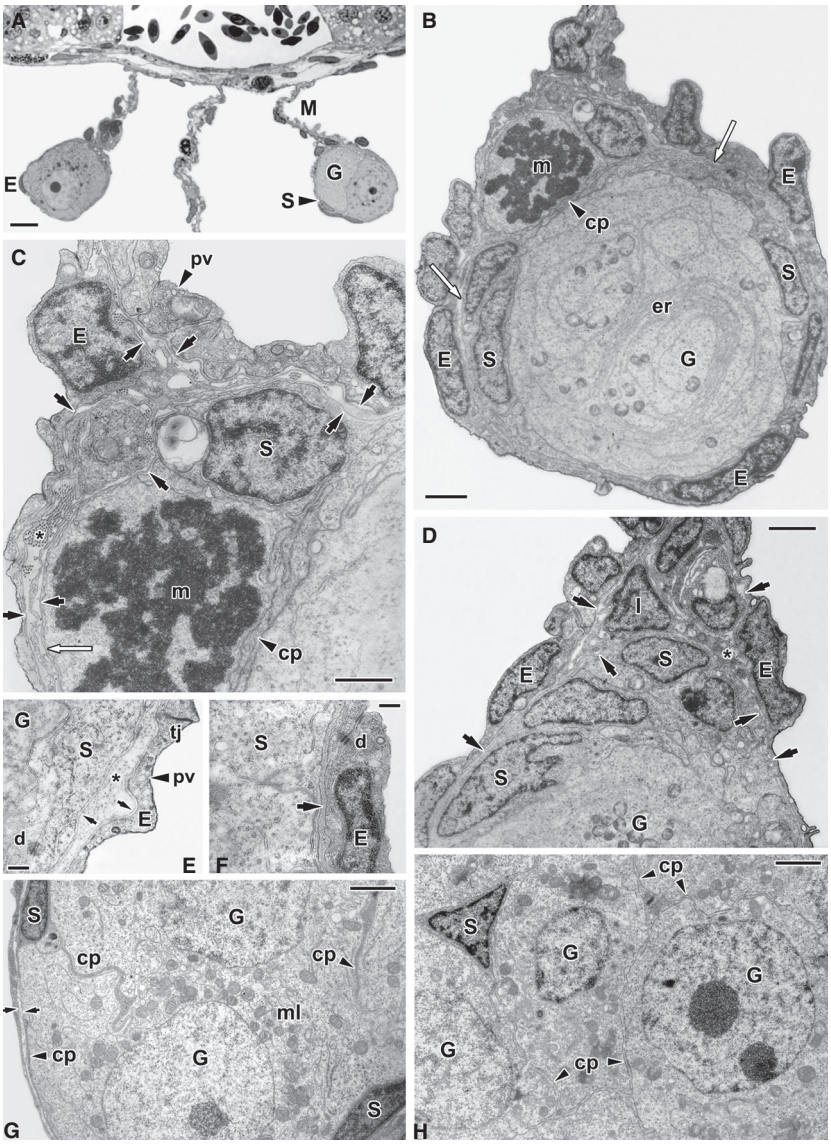

Fig. 1—Undifferentiated gonads of *Cichlasoma dimerus* on days 14 (A–F) and 35 (G, H) postfertilization. A, Light microscopy (LM) (semithin section). B–H, Transmission electron microscopy (TEM). —**A.** Cross section of gonadal primordia suspended from the dorsal peritoneum by short mesenteries. —**B.** Cross section of the gonadal primordium in which the different cell types are observed. A mitotic figure is evidenced in one of the germ cells. A thin interstitial compartment (white arrows), containing collagen fibrils, is observed between epithelial and support cells, except in the ventral side of the gonad, where an epithelial cell comes into direct contact with a germ cell (reprinted from Meijide *et al.* 2005). —**C.** Area from B, showing the spatial relations between the basement membrane and the different cell types. Note how the basement membrane separates the germinal and interstitial compartments of the gonad. Germ cells are encompassed by the cytoplasm of support cells but occasionally lie in direct contact with the basement membrane, as observed in this image (white arrow). —**D.** Dorsal region of the gonadal primordium showing the different types of somatic cells. —**E.** Detail of the peripheral region of the gonadal primordium. Note the presence of a 'double' basement membrane (arrows) corresponding to a folding of the epithelial basement membrane. The outer basement membrane supports peripheral epithelial cells; the inner basement membrane subtends internal support cells that are associated with germ cells. Collagen fibrils, which are indicative of the interstitial compartment, are observed between both basement membranes. —**F.** Detail of a region where the 'two' basement membranes fuse so that they are observed as a single thick membrane (arrow). —**G.** Upon mitotic division, germ cells may undergo kariokinesis without cytokinesis so that a cytoplasmic continuity is observed between adjacent nuclei (note the absence of plasma membranes in between). Separation of the cytoplasm is achieved as cytoplasmic extensions of support cells grow between the nuclei. —**H.** Cytoplasmic processes of support cells encompass germ cells, separating one from another. Scale bars: (A) 5 μm, (B, D, G, H) 2 μm, (C) 1 μm, (E, F) 0.2 μm. arrow, basement membrane; asterisk, collagen fibrils; cp, cytoplasmic process; d, desmosome; E, epithelial cell; er, smooth endoplasmic reticulum; G, germ cell; I, interstitial mesenchymal cell; M, gonadal mesentery; m, mitotic figure; ml, mitochondria with lamellar cristae; pv, pinocytotic vesicles; S, support cell; tj, tight junction.

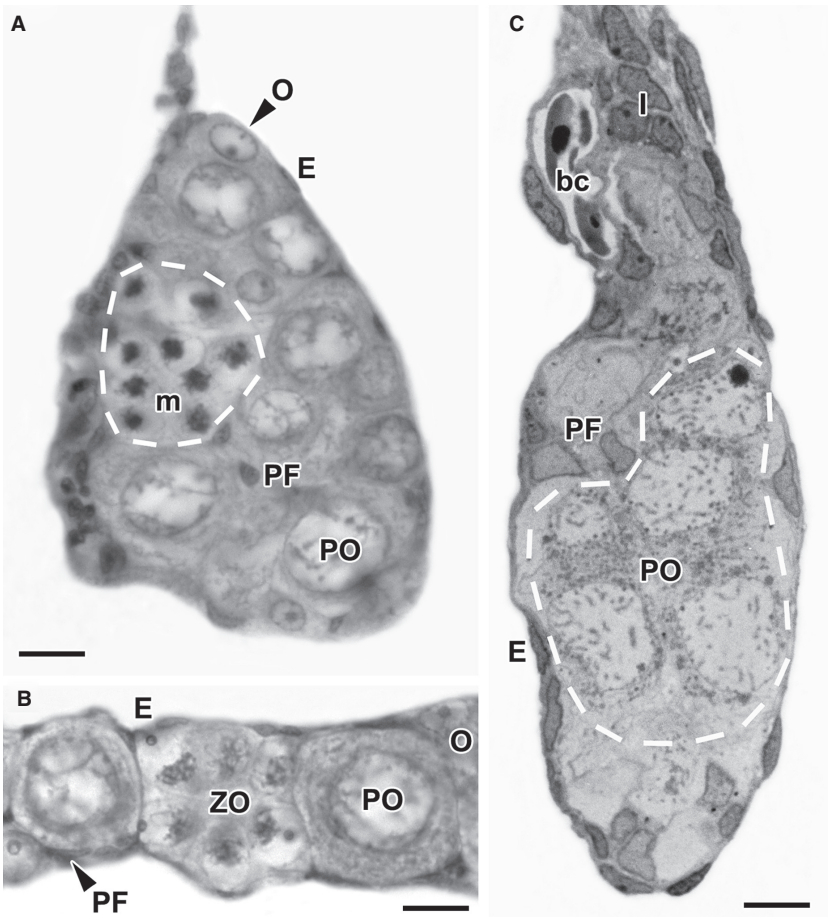

Fig. 2—Ovarian differentiation in *Cichlasoma dimerus* on days 38 to 45 postfertilization. LM. A–B, Paraffin sections. C, Semithin section. A–C: Ovarian differentiation is marked by increased germ cell mitosis and the subsequent onset of meiosis. Mitotic cystocytes enter meiosis synchronously giving rise to cysts of early prophase oocytes. Synaptonemal complexes are observed as strands of condensed chromatin inside the nuclei of zygotene and pachytene oocytes. —**A.** Cross section of the ovary showing synchronous mitotic figures appearing in groups, which are interpreted as a cyst-forming type of division (the mitotic germline cyst is encircled by the dotted line). —**B.** Longitudinal section showing a meiotic germline cyst of zygotene oocytes, without intervening prefollicle cells. The cyst is flanked by two pachytene oocytes that are individually enveloped by prefollicle cells, forming follicles. Note the bouquet arrangement of chromosomes in nuclei at the zygotene step of meiotic prophase. —**C.** Cross section showing a germline cyst of pachytene oocytes (dotted line). Prefollicle cells observed at the periphery of the cyst do not interpose between the interconnected oocytes. Scale bars: 10 μm. bc, blood capillary; E, epithelial cell; I, interstitial mesenchymal cell; m, mitotic figure; O, oogonium; PF, prefollicle cell; PO, pachytene oocyte; ZO, zygotene oocyte.

chromosomes take place (Fig. 2A–C). EM examination reveals that the oocytes within a cyst remain interconnected by intercellular bridges, this cytoplasmic communication enabling synchronous development (Fig. 3B,C). In certain sections, a broader cytoplasm communication is observed between adjacent nuclei, because cell limits appear to be absent in between (Fig. 3D). At the pachytene step of meiosis, cytoplasmic processes of prefollicle cells grow between the oocytes (Fig. 3E), which become isolated from each other and continue their development in individual follicles. Upon formation of the follicle, the oocyte progresses to diplotene and initiates the primary growth (Fig. 4A). In the cytoplasm of meiotic oocytes, paired centrioles known as diplosomes are occasionally observed (Fig. 3G). Within the interstitial compartment, mesenchymal cells become more numerous than in previous stages of development. These cells arise from the connective tissue subjacent to the coelomic epithelium and invade the gonad primordium to form the ovarian stroma; along with collagen fibrils, they are observed in the dorsal and peripheral regions of the gonad, lying between epithelial and prefollicle cells (Figs 2C and 3A,F).

The ovarian lumen is formed towards days 50–65. Initially, somatic cells from the dorsal and ventral edges of the ovary proliferate and form appendix-like structures that protrude from the gonad (Fig. 4A). Subsequently, these outgrowths form thin sheets that face each other (Fig. 4C) and finally fuse (Fig. 4B) to form the characteristic lumen of an ovary of the cystovarian type. The examination of different regions of the ovary along its longitudinal axis reveals that formation of the lumen progresses in antero-posterior direction (Fig. 4B–D). Occasionally, germ cells migrate towards the recently formed laminar tissue (Fig. 4E). By this stage of development, numerous follicles containing primary growth oocytes are present. The cytoplasm of these cells becomes highly basophilic, and lampbrush chromosomes are visualized as thin chromosome threads that form a random reticulum within the nucleoplasm (Fig. 4). With the onset of primary growth, oocytes become bigger and as a result, the size of the ovary increases significantly.

The germinal epithelium in the differentiated ovary

From day 80 to 90 onwards, many developing follicles, as well as younger oocytes at early steps of meiotic prophase and oogonia, are observed in the ovary (Fig. 5A,B). Oogonia and early prophase oocytes reside in the luminal edge of the developing ovarian lamellae; along with the epithelial and prefollicle cells, they form the so-called germinal epithelium

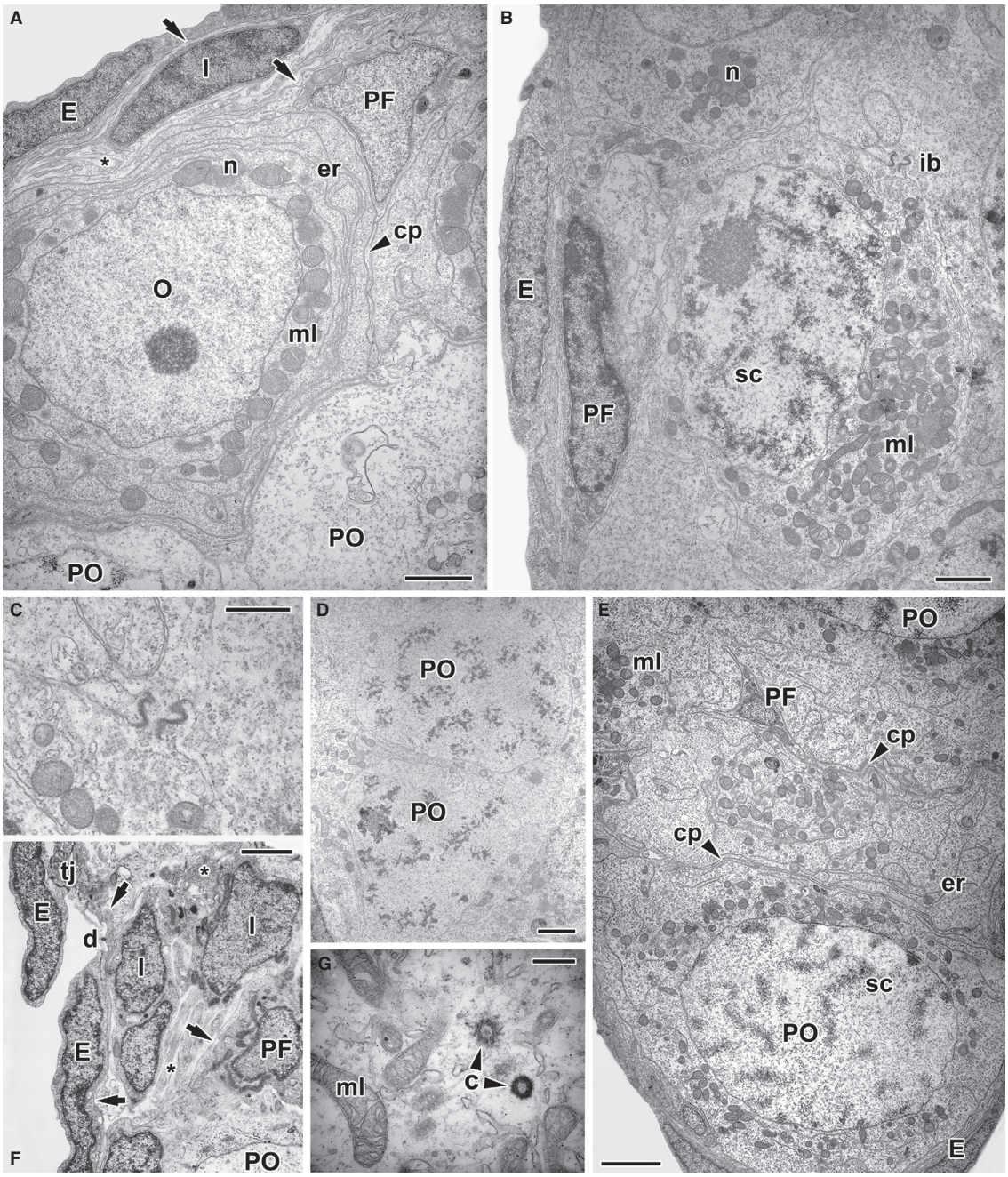

Fig. 3—Ovarian differentiation in *Cichlasoma dimerus* on days 38 to 45 postfertilization. TEM. —**A.** An oogonium is encompassed by cytoplasmic processes of a prefollicle cell (reprinted from Meijide *et al.* 2005). —**B.** Chromatin nucleolus oocyte in the pachytene step showing synaptonemal complexes within the nucleoplasm. An intercellular bridge connects the oocyte to another one inside the same cyst. —**C.** Area from B showing the intercellular bridge connecting pachytene oocytes at higher magnification. —**D.** In certain sections, an absence of cell membranes is evidenced between adjacent pachytene nuclei. This might be the result of the absence of cytokinesis following the cyst-forming division, before the nuclei enter meiosis. —**E.** In germline cysts containing pachytene oocytes, the surrounding prefollicle cells move towards the interior and progressively encompass individual oocytes with cytoplasmic extensions. —**F.** Dorsal region of the differentiating ovary. Interstitial mesenchymal cells and collagen fibrils become more numerous than in the undifferentiated gonad. —**G.** Paired centrioles called diplosomes are occasionally observed in the cytoplasm of early prophase oocytes. Scale bars: (D, E) 3 μm, (A, B, F) 2 μm, (C) 1 μm, (G) 0.2 μm. arrow, basement membrane; asterisk, collagen fibrils; c, centrioles; cp, cytoplasmic process; d, desmosome; E, epithelial cell; er, smooth endoplasmic reticulum; I, interstitial mesenchymal cell; ib, intercellular bridge; ml, mitochondria with lamellar cristae; n, nuage; PF, prefollicle cell; PO, pachytene oocyte; sc, synaptonemal complex; tj, tight junction.

(Figs 5A–C and 6A–C,E), which is separated from the ovarian stroma by the subtending basement membrane (Fig. 6B, C,E–G). Within the epithelium, oogonia and early oocytes occur in clusters known as cell nests (Figs 5B,C and 6C,E). Oogonia are individually surrounded by cytoplasmic processes of prefollicle cells (Fig. 6C,E) and may undergo self-renewing mitotic divisions. Alternatively, an oogonium may divide mitotically with incomplete cytokinesis producing a cluster of cystocytes that synchronously enter meiosis. The germline cyst thus originated is surrounded by peripheral prefollicle and epithelial cells (Figs 5C and 6C). Initially, no cytoplasm of prefollicle cells is observed between the cystic oocytes (Fig. 6C), which remain interconnected by intercellular bridges (Fig. 6D). At the pachytene step of meiotic prophase, cytoplasmic extensions of prefollicle cells grow between the oocytes, individualizing them and initiating the formation of follicles (Fig. 6B). Intercellular bridges disappear, and the germline cyst is broken down. As folliculogenesis progresses, the ovarian follicles tend to separate from the cell nest. Concomitantly, the oocytes advance in prophase I, becoming arrested in diplotene and initiating the primary growth (Fig. 5C). Recently formed follicles are composed of a characteristically large, perinucleolar oocyte and the surrounding follicle cells and remain segregated

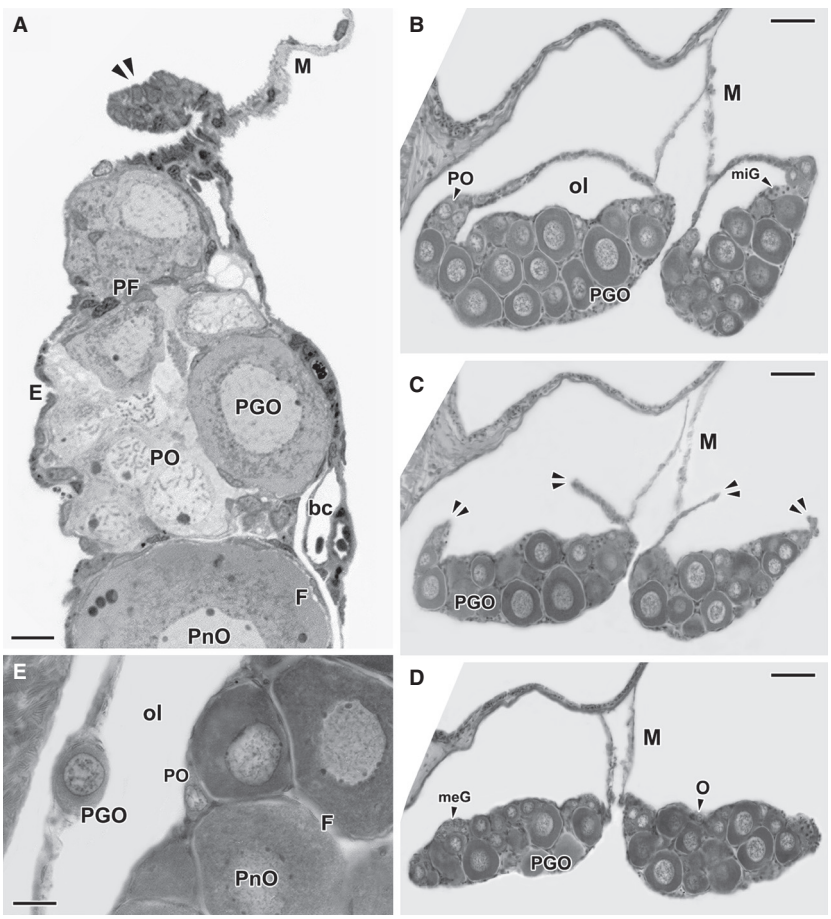

Fig. 4—Somatic reorganization and formation of the ovarian lumen in *Cichlasoma dimerus* between days 50 and 65 postfertilization. LM. A, Semithin section. B–E, Paraffin sections. —**A.** Cross section of the ovary on day 50 showing cystic pachytene oocytes and follicles containing oocytes at different steps of primary growth. An outgrowth of somatic cells (double arrowhead) is observed in the dorsal edge of the gonad. A laminar tissue will develop from these cells, as well as from a ventral outgrowth, and the ovarian lumen will be formed as these appendix-like expansions fuse. —**B–D.** The ovarian cavity is formed following an antero-posterior gradient, as evidenced in serial sections of the ovary in day 65 postfertilization. In an anterior cross section, the lumen is formed (B). In a middle section, somatic outgrowths are observed (double arrowheads) (C). In a posterior section, somatic outgrowths are absent (D). —**E.** When the ovarian lumen is completely formed on day 65, many follicles formed by a perinuleolar oocyte and surrounding follicle cells are observed. Occasionally, early-stage germ cells migrate into the laminar tissue that develops during the formation of the ovarian cavity. Scale bars: (B–D) 50 μm, (A, E) 10 μm. bc, blood capillary; E, epithelial cell, F, follicle cell; meG, meiotic germline cyst; miG, mitotic germline cyst; M, mesovary; O, oogonium; ol, ovarian lumen; PF, prefollicle cells; PGO, primary growth oocyte; PnO, perinucleolar oocyte; PO, pachytene oocyte.

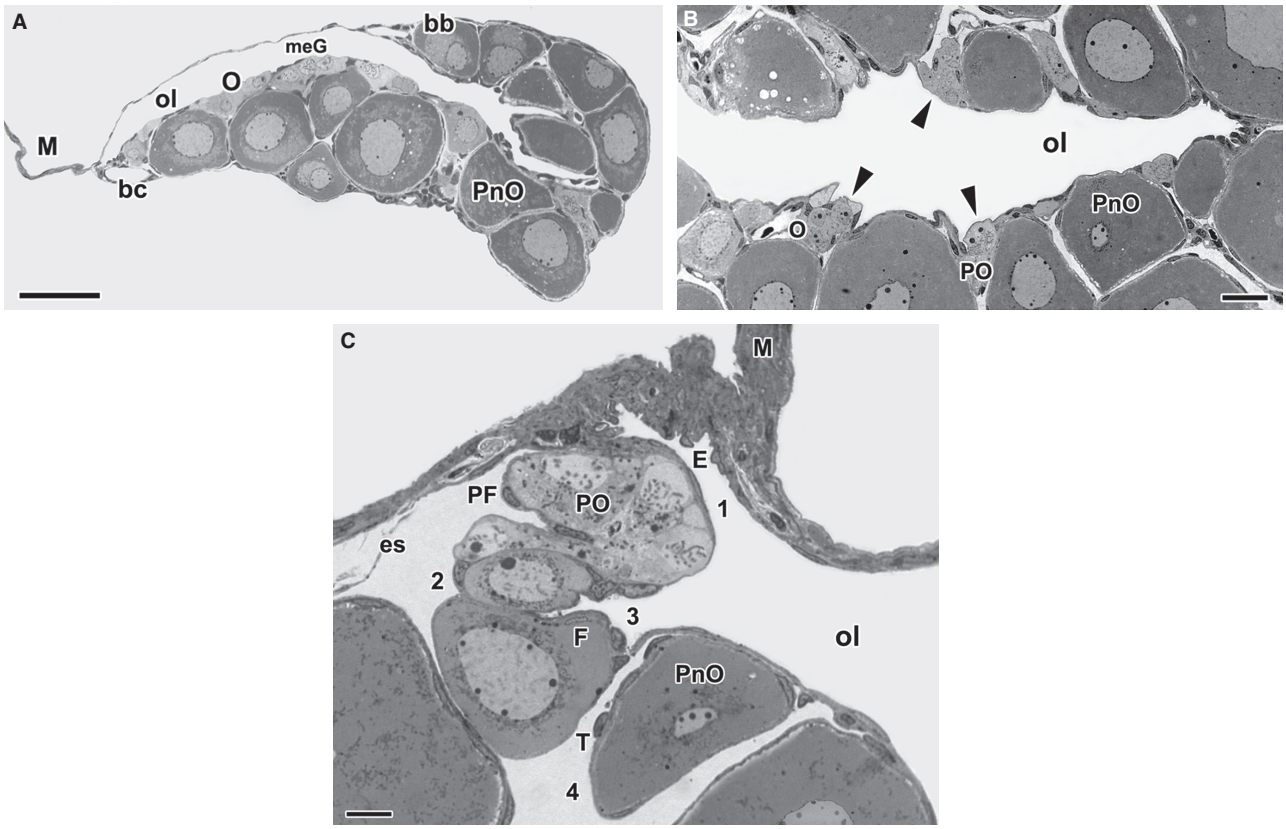

Fig. 5—Ovarian development in *Cichlasoma dimerus* on day 90 postfertilization. LM (semithin sections). —**A.** Cross section of the ovary showing numerous follicle complexes with primary growth oocytes at the perinucleolar step. The lighter areas within the basophilic correspond to aggregations of membranous organelles known as Balbiani bodies. —**B.** Detail of the germinal epithelium lining the ovarian lumen. Nests of early-stage germ cells (arrowheads) occur within inpockets along its length. Follicle complexes contacting the germinal epithelium are observed between cell nests. —**C.** Detail of a region of the germinal epithelium in which a representative sequence of follicle complex development is observed. (1) A germline cyst of pachytene oocytes is part of a cell nest within the germinal epithelium. Prefollicle and epithelial cells are observed at the periphery of the cyst and do not intermingle with the oocytes. (2) Within the cell nest, an oocyte that has already entered the primary growth stage is enveloped by cytoplasmic extensions of prefollicle cells. The cytoplasmic basophilia increases within the oocyte. (3) A recently formed ovarian follicle, composed of a perinucleolar oocyte and the surrounding follicle cells, protrudes into the stroma as it leaves the cell nest. (4) Thecal cells encompass the basement membrane outside the follicle, thus forming a follicle complex. Scale bars: (A) 50 μm, (B) 20 μm, (C) 10 μm. bb, Balbiani body; bc, blood capillary; E, epithelial cell; es, extravascular space; F, follicle cell; M, mesovary; meG, meiotic germline cyst; O, oogonium; ol, ovarian lumen; PF, prefollicle cell, PnO, perinucleolar oocyte; PO, pachytene oocyte; T, thecal cell.

from the stroma by a basement membrane that is synthesized by prefollicle cells during folliculogenesis (see Mazzoni *et al.* 2010) (Figs 5C and 6A,B, E–G). Oocytes in the perinucleolar step of primary growth are characterized by having multiple round nucleoli in the peripheral nucleoplasm and a basophilic cytoplasm (Figs 5A–C and 6A). In the larger oocytes, the cytoplasm assumes a fissured appearance due to the proliferation of membranous organelles. These areas correspond to the mitochondrial clouds or Balbiani bodies, which are distinguished around the nucleus, where they become polarized towards the vegetal hemisphere (Fig. 5A). Few squamous follicle cells (the former prefollicle cells) flatten out over the surface and completely surround each growing oocyte. Microvilli are formed by folding of the oolemma and extend from the oocyte surface, intermingling with those of the overlying follicle cells (Fig. 6F,H). Mesenchymal cells from the stroma begin to encompass each follicle and its surrounding basement membrane (Fig. 6E), giving rise to the theca (Fig. 6F,G). The follicle, basement membrane and theca form a follicle complex (Figs 5C and 6A). Both follicle and thecal cells have little cytoplasm that contains few organelles. Between follicles, an area known as the stromal extravascular space becomes evident, in which mesenchymal and the derived thecal cells, collagen fibrils and blood capillaries reside (Figs 5C and 6A,B,G,H). When folliculogenesis is completed, the newly formed ovarian follicle remains connected to the germinal epithelium via a shared portion of basement membrane, that is a single basement membrane is present at the point where the follicle complex is attached to the germinal epithelium (Fig. 6H,I).

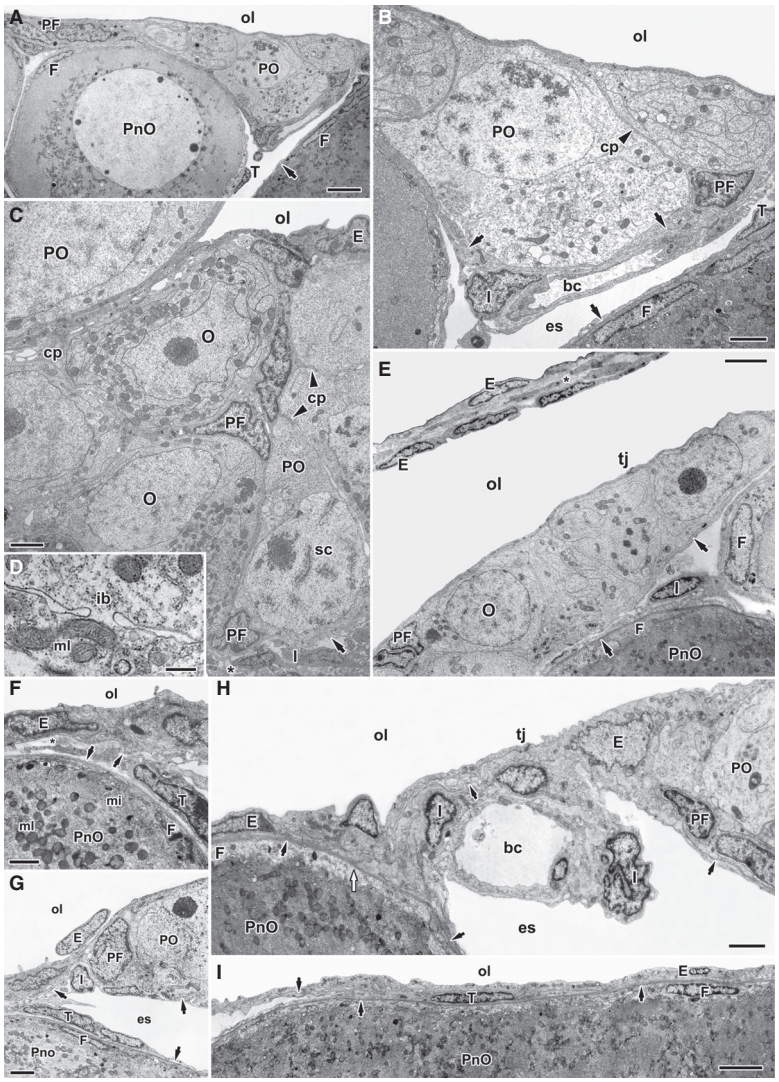

Fig. 6—The ovarian germinal epithelium in *Cichlasoma dimerus* at day 90 postfertilization. TEM. —**A.** Pachytene oocytes and associated prefollicle cells occur within the germinal epithelium. An adjacent follicle, which arose from the germinal epithelium, is composed of a perinucleolar oocyte and the encompassing follicle cells. The follicle along with the surrounding basement membrane and thecal cell layer form a follicle complex. —**B.** Area from A, showing a pachytene oocyte within the germinal epithelium. At the beginning of folliculogenesis, cytoplasmic processes of prefollicle cells individualize this oocyte from the adjacent ones. —**C.** Detail of a germ cell nest within the germinal epithelium. Non-cystic oogonia and a germline cyst of pachytene oocytes occur together within the nest. Oogonia are individually enveloped by prefollicle cells. Cytoplasmic extensions of prefollicle cells located at the periphery of the cyst are just beginning to grow between the oocytes. —**D.** Detail of an intercellular bridge connecting two oocytes within the cyst. —**E.** The ovarian lumen is bordered by the germinal epithelium 'at one side' and by a double layer of epithelial cells with few connective tissue elements in between 'at the opposite side'. An interstitial mesenchymal cell from the ovarian stroma contacts the basement membrane that surrounds a follicle, giving rise to the theca. —**F.** In recently formed follicles, formation of microvilli by folding of the oocyte and follicle cell plasma membranes is evidenced. —**G.** Detail of the germinal epithelium and an adjacent follicle complex. Interstitial cells residing within the stromal extravascular space are located below the epithelial basement membrane. —**H.** Detail of the germinal epithelium and underlying stroma, which are separated by the epithelial basement membrane. On the left, a developing follicle is attached to the germinal epithelium, as they share a common portion of basement membrane. Note how the basement membrane that supports the germinal epithelium becomes 'one' with that of the follicle at the point of attachment (white arrow). —**I.** Detail of the area of attachment of a follicle complex to the germinal epithelium. The theca is formed except in the region where the basement membrane subtending the germinal epithelium fusions with that of the follicle complex. Scale bars: (A) 5 μm, (E, I) 3 μm, (B, C, G) 2 μm, (F, H) 1 μm, (D) 0.5 μm. arrow, basement membrane; asterisk, collagen fibrils; bc, blood capillary; c, cytoplasmic process; E, epithelial cell; es, extravascular space; F, follicle cell; I, interstitial mesenchymal cell; ib, intercellular bridge; mi, microvilli; ml, mitochondria with lamellar cristae; O, oogonium; ol, ovarian lumen; PF, prefollicle cell; PnO, perinucleolar oocyte; PO, pachytene oocyte; sc, synaptonemal complex; T, thecal cell; tj, tight junction.

Schematic summary of the morphogenesis of the ovarian germinal epithelium

Formation of the germinal epithelium is schematically depicted in Fig. 7. In the undifferentiated gonadal primordium, germ cells, along with their supporting cells, initially occur within an inpocketing of the coelomic epithelium that circumscribes the gonad, as indicated by a continuous underlying basement membrane that folds into the pocket. Epithelial cells, support cells and germ cells form the primordial germinal epithelium and represent the germinal compartment of the gonadal anlage. Cells belonging to the germinal compartment are segregated from the interstitial compartment by a basement membrane right from this early stage of gonadal development. Initially, the gonadal interstitium contains collagen fibrils and few dorsally located mesenchymal cells (Fig. 7A). Ovarian differentiation is signalled by increased germ cell mitosis and the onset of meiosis. In the recently differentiated ovary, oogonia, early meiotic oocytes and the encompassing prefollicle cells reside within an inpocketing of the surface epithelium, as in the undifferentiated gonad. Oogonia dividing by mitosis remain interconnected by intercellular bridges forming germline cysts; the cystocytes then enter meiosis becoming oocytes that develop synchronously up to the pachytene step of meiotic prophase. Each germline cyst is surrounded by peripheral prefollicle cells. Subsequently, cytoplasmic processes of prefollicle cells grow between the oocytes, isolating them and giving rise to individual follicles (Fig. 7B). By the time the ovarian lumen begins to form, some of the oocytes have reached the perinucleolar step of the primary growth, their cytoplasm becoming typically basophilic. These oocytes are no longer located within an inpocketing of the germinal epithelium. They occur within follicles that originated from it. Each ovarian follicle is formed by a perinucleolar oocyte and the encompassing follicle cells and is surrounded by a basement membrane. Mesenchymal cells from the stroma form the theca around the basement membrane (Fig. 7C). The somatic outgrowths that enclose part of the coelom and form the ovarian lumen are composed of a double layer of epithelial cells with few stromal connective tissue elements in between. As these outgrowths fuse, the resulting ovarian lumen becomes internally lined by the former coelomic (germinal) epithelium, which will develop as the typical germinal epithelium bordering the ovigerous lamellae (Fig. 7D). The outer surface of the ovary is covered by the coelomic epithelium (mesothelium), which is continuous with that of the mesovary. The germinal epithelium contains epithelial cells and derived prefollicle cells that become associated with nested early-stage germ cells within several inpockets along its length. In these germ cell nests, an oogonium may divide by mitosis forming new oogonia. Usually, it undergoes several rounds of divisions with incomplete cytokinesis, giving rise to a group of interconnected cystocytes that synchronously enter meiosis. The germline cyst thus formed is encompassed by peripheral epithelial and prefollicle cells. Folliculogenesis is initiated as the oocytes at pachytene become individually enclosed by cytoplasmic processes of prefollicle cells. The oocyte and associated prefollicle cells begin to descend from the surface of the germinal epithelium, protruding into the stroma. At this point, the oocyte becomes arrested at diplotene and initiates primary growth. Folliculogenesis is ended when the oocyte at the perinucleolar step and its encompassing prefollicle cells become completely surrounded by a basement membrane which segregates the follicle from the stroma. Then prefollicle cells become follicle cells. The basement membrane around the forming follicle closes off but does not completely separate it from the germinal epithelium; rather, the follicle remains attached to the germinal epithelium as both share a short portion of basement membrane. At the same time, mesenchymal cells from the stroma form the theca around the follicle (except in the region where the follicle is attached to the germinal epithelium), and thus, the follicle complex is formed (Fig. 7D).

Discussion

Initial development of the germinal epithelium

In teleost fishes, the genital ridges form as longitudinal thickenings of mesoderm that protrude into the coelomic cavity ventral to the developing kidney and lateral to the dorsal mesentery (Devlin and Nagahama 2002). Primordial germ cells arising at extragonadal sites migrate to the genital ridges and colonize it (Braat *et al.* 1999). Once within the genital ridge, primordial germ cells can be identified as conspicuously large cells within a modified layer of proliferating mesothelium, this tissue being referred to as 'germinal epithelium' (*sensu* Hoar 1969). The literature regarding the initial development of the teleost gonad is confusing and sometimes contradictory. In the medaka, *Oryzias latipes*, the gonadal anlage was reported to consist only of coelomic epithelium encompassing primordial germ cells (Hamaguchi 1982, 1992). A review by Devlin and Nagahama (2002) states that, prior to gonad differentiation, all somatic cells appear to be derived from a cortex epithelial layer; however, some somatic cells might arise from the invasion of mesenchyme. In turn, Le Menn *et al.* (2007) argue that the undifferentiated Teleostei gonad consists of a conjunctive stroma containing germ cells, as can be seen in the golden grey mullet, *Liza aurata*. The ultrastructural evidence from the present study indicates that the somatic component of the gonadal primordium is represented by two cell types residing in distinct tissue compartments: epithelial cells and the derived support cells arise from mesothelial cells of the coelomic epithelium; interstitial cells are derived from mesenchymal cells belonging to the subepithelial connective tissue. A developing basement membrane separates the epithelial and the interstitial compartments of the gonad right from an initial stage of development. Support cells become follicle cells in females and Sertoli cells in males, as proposed for *O. latipes* by Hamaguchi (1992) and reported for *Cichlaso-*

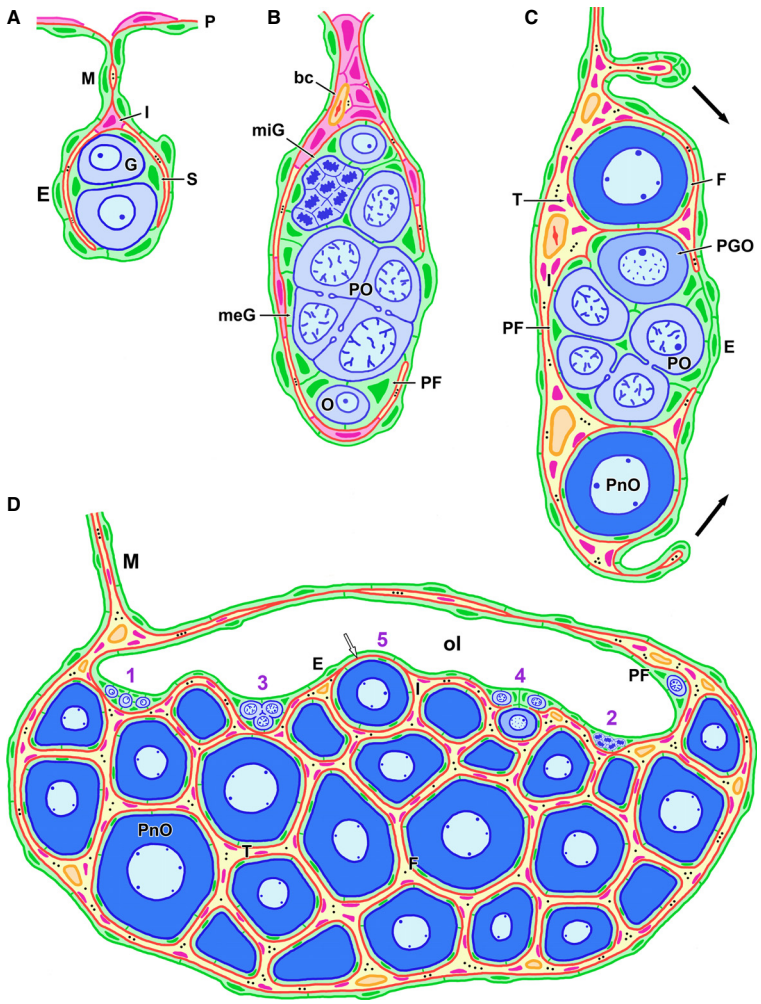

Fig. 7—Diagrammatic representation illustrating the development of the germinal epithelium and the process of folliculogenesis during ovarian morphogenesis in *Cichlasoma dimerus*. Note the spatial relations between different cell types and the basement membrane. As the gonadal primordium forms, the mesothelial basement membrane extrudes from the peritoneum into the gonad, separating the germinal and interstitial compartments from the beginning of gonadal development (A). In the undifferentiated gonadal primordium (A) and recently differentiated ovary (B), early-stage germ cells and the accompanying support cells occur within an inpocketing of the peripheral coelomic epithelium. Somatic outgrowths containing both epithelial and mesenchymal cells enclose part of the coelom (C) and form the ovarian lumen, which results lined internally by the former coelomic epithelium (D). Once the ovarian lumen is formed, the ovary contains numerous developing follicles with primary growth (perinucleolar) oocytes. These follicles are derived from the germinal epithelium and remain connected to it at some point along the follicle surface, as revealed by a single shared basement membrane (white arrow). This is the site where the oocyte will be released during ovulation. Oogonia and early prophase oocytes form cell nests within inpockets of the germinal epithelium, where they become associated with prefollicle cells (D). 1–5 illustrates different stages of early oogenesis and follicle complex development. Oogonia residing in the germinal epithelium (1) undergo cyst-forming mitotic divisions (2), giving rise to a cluster of cells that synchronously enter meiosis. The cystic oocytes are connected by intercellular bridges and surrounded by peripheral epithelial and prefollicle cells (3). At the onset of folliculogenesis, cytoplasmic processes of prefollicle cells individually encompass each oocyte. Subsequently, the oocyte and associated prefollicle cells become enclosed within a basement membrane that extends up over the forming follicle and tends to separate it from the germinal epithelium (4). Mesenchymal cells from the ovarian stroma become associated with the basement membrane that surrounds the developing follicle and form the theca. The follicle complex, which is formed by the follicle, basement membrane and theca, remains attached to the germinal epithelium through a shared portion of basement membrane (5). Colours. Green: cells of epithelial origin. Blue: oogonia and oocytes. Purple: interstitial mesenchymal cells. Black: collagen fibrils. Orange: blood vessels. Yellowish-cream: extravascular space. Red: basement membrane. Cell limits of interstitial cells were not drawn in C and D for clarity. This diagram was not drawn completely to scale. The process depicted in this drawing might be generalized for most teleosts. bc, blood capillary; E, epithelial cell; F, follicle cell; G, germ cell; I, interstitial mesenchymal cell; M, gonadal mesentery/mesovary; meG, meiotic germline cyst; miG, mitotic germline cyst; O, oogonium; ol, ovarian lumen; P, peritoneum; PF, prefollicle cell; PGO, early primary growth oocyte; PnO, perinucleolar oocyte; PO, pachytene oocyte; S, support cell; T, thecal cell.

ma dimerus in a previous study (Meijide *et al.* 2005). More recently, *sox9b*-expressing cells were identified in *O. latipes* as common precursors of the supporting cells of both sexes, which later differentiate into Sertoli or follicle cells (Nakamura *et al.* 2008). This is coincident with the standard model of mammalian sex differentiation in which the Sertoli and granulosa (follicle) cells directly stem from the supporting cell precursors of the bipotential gonad (Mork *et al.* 2012). Regarding their embryonic origin, these cells are considered to derive from the coelomic epithelium, that is from the lateral plate mesoderm (Gilbert 2003). In the case of the interstitial cells, the literature is far from being conclusive. Even in mammals, little is known about the origin of the interstitium or the cellular diversity within the gonadal early stromal compartment (De Falco *et al.* 2011). Generally, it is accepted that interstitial mesenchymal cells differentiate into thecal cells in the ovary and Leydig cells in the testis. We note that the term mesenchyme is rather vague in terms of embryonic origin and whether these cells are derived from the intermediate mesoderm or the lateral plate mesoderm remains to be determined. Furthermore, an origin from an embryonic layer other than the mesoderm might be possible because a hypothesis suggests that Leydig stem cells derive from the neural crest (Lejeune *et al.* 1998), and thecal cells, as the female counterpart, might share the same origin.

Formation of the germinal epithelium during ovarian morphogenesis has been previously addressed in the common carp, *Cyprinus carpio* (see Mazzoni *et al.* 2010). However, a coherent interpretation of the initial development of the germinal epithelium, compatible with observations from previous studies performed in the same species, is lacking in that study. Although Mazzoni *et al.* (2010) maintain that peripheral somatic cells of the gonadal primordium form a continuum with the coelomic epithelium, neither epithelial cells nor a basement membrane are illustrated in their scheme of a 7–37 days postfertilization gonadal primordium. Likewise, no distinction is made between the different types of somatic cells that surround germ cells in the gonadal primordium. This might have been the result of an incomplete ultrastructural analysis of the initial stages of gonadal development. In a first histological study carried out by Ryazantseva and Sakum (1980), germ cells at day 9 after hatching were reported to be surrounded by somatic cells of the coelomic epithelium. Coincidently, at day 7 postfertilization, Parmentier and Timmermans (1985) described the presence of cyst (support) cells associated with germ cells, these cells forming a continuous layer with peritoneal (mesothelial) cells. Later, at day 28, stromal cells were recognized, although the origin of these cells was unclear. According to van Winkoop *et al.* (1992), upon arriving at the genital ridge, primordial germ cells are ensheathed by cells derived from the gonadal epithelium. And at 35 days postfertilization, two layers of enveloping somatic cells can be ultrastructurally recognized within the gonadal primordium of *C. carpio*: lighter central cells, located close to germ cells, and peripheral cells, both resting on basement membranes with connective tissue in between. These observations on the gonadal primordium of *C. carpio* are compatible with the cell architecture we describe here (see Fig. 7A), in which the cells from the first two studies have been referred to as support and interstitial cells, respectively, the former being derived from the coelomic epithelium and the latter from the subjacent mesenchymal tissue. The cells described in the third study were herein designated as epithelial and support cells, respectively. In this sense, the gonadal primordium of *C. carpio* at 4–5 weeks (van Winkoop *et al.* 1992) is comparable to that of *C. dimerus* at 2–5 weeks (present study), for the presence of peripheral epithelial cells, internal support cells that surround germ cells, and a thin interstitial tissue containing collagen fibrils and few mesenchymal cells in between.

In the scheme of germinal epithelium formation proposed for *C. carpio* by Mazzoni *et al.* (2010), germ cells appear in an interior position within the differentiating ovary, associated with somatic cells and surrounded by a basement membrane that is not in contact with that of the surface epithelium. As a result, 'two basement membranes' are observed between epithelial and support cells. A possible explanation to this feature, as suggested by Mazzoni *et al.* (2010), is that germ cells and the associated support cells initially become internalized within the genital ridge, losing contact with the surface epithelium, and later, as ovarian lamellae are formed by invaginations of the epithelium, germ cells are incorporated into the epithelial cell layer, thus establishing a germinal epithelium. The interpretation proposed in the present study is different in the sense that germ cells, support cells and epithelial cells are depicted to be part of tissue continuum, with the epithelial basement membrane being folded to form an inpocket within which germ cells and support cells reside. Thus, germ cells are part of a germinal epithelium right from the beginning of gonadal development. This interpretation is consistent with germ cells migrating to the genital ridge through the dorsal gut mesentery, then laterally along the coelomic epithelium (Patiño and Takashima 1995; Devlin and Nagahama 2002), where they first make contact with their supporting cells (Hamaguchi 1992), and residing within the germinal epithelium upon arrival to the genital ridge (as proposed in this study); also it is supported by the architecture found in the adult syngnathid ovary (see Selman *et al.* 1991) as discussed later. It should be noted, however, that the scheme proposed for *C. carpio* would be similar to the one depicted in the present study if a continuum were evidenced at some point between the basement membrane subtending the surface epithelium and that surrounding germ cells and the associated support cells. In fact, this connection of the basement membranes may be frequently not observed due to the plane of sections, so that both interpretations are possible. During the ovarian differentiation of *Siganus guttatus*, sex cord-like structures delimited by a basement membrane and containing germ cells and associated somatic cells, similar to those described in *C. carpio*, were depicted to be in contact with the surface epithelium and later with the luminal epithelium

(Komatsu *et al.* 2006), similarly to *C. dimerus*. Probably, further analysis is required in more species to elucidate the way the ovarian germinal epithelium develops in teleosts.

Germ cell proliferation and formation of germline cysts

Upon colonizing the gonadal anlage, germ cells increase in number due to mitotic proliferation (Patiño and Takashima 1995; Braat *et al.* 1999). Interestingly, the present study revealed an unusual pattern of germ cell division. When germ cells divide mitotically, some of them seem to undergo kariokinesis without cytokinesis, rendering a temporary cytoplasmic continuity, with absence of cell membranes between adjacent nuclei. Separation of the cytoplasm is afterwards facilitated by the growth of processes of the accompanying support cells. As a first recognizable sign of gonadal sex differentiation in *C. dimerus*, germ cells proliferate at a higher rate in the presumptive ovaries, as reported in *O. latipes* (see Satoh and Egami 1972; Hamaguchi 1982). In this species, two types of germ cell proliferation were demonstrated. Type I proliferation involves germ cell self-renewal and produces isolated daughter cells. Type II is a gametogenic, clonal mode of proliferation where gonial divisions form cysts of interconnected cells that synchronously enter meiosis (Saito *et al.* 2007). In a similar fashion to *O. latipes*, germ cells in the undifferentiated gonads of *C. dimerus* proliferate by intermittent type I division. While in the presumptive testes germ cells continue this mode of proliferation, ovarian differentiation is signalled by an early initiation of type II divisions leading to the formation of cysts in which germ cells synchronously enter meiosis and start to develop as oocytes. However, according to Marlow (2010), the observation of divisions producing individual oocytes without forming cysts indicates that interconnections (type II divisions) are not absolutely essential for oocyte specification. Here, intercellular bridges were observed as cytoplasmic connections between early prophase oocytes forming a cyst, as evidenced in other teleosts (Le Menn *et al.* 2007; Saito *et al.* 2007; Mazzoni *et al.* 2010; Quagio-Grassiotto *et al.* 2011).

In teleosts, formation of germline cysts was reported both in the ovaries during differentiation (Saito *et al.* 2007; Mazzoni *et al.* 2010; present study) and as part of oogenesis in the adult ovaries (Le Menn *et al.* 2007; Quagio-Grassiotto *et al.* 2011; Wildner *et al.* 2013). Germline cysts are formed when oogonia divide mitotically with incomplete cytokinesis giving rise to a cluster of interconnected germ cells that synchronously enter meiosis, becoming oocytes. They disappear as the oocytes in pachytene of the first meiotic prophase (Matova and Cooley 2001) are individualized by cytoplasmic processes of prefollicle cells that grow between them during folliculogenesis (Mazzoni *et al.* 2010; Quagio-Grassiotto *et al.* 2011), as observed in *C. dimerus*. Germline cysts have been reported to contribute to oogenesis in other animal species (de Cuevas *et al.* 1997; Pepling *et al.* 1999; Matova and Cooley 2001; Kloc *et al.* 2004). In the mouse, cysts are formed following the arrival of primordial germ cells at the genital ridge during embryonic development. Cystocytes then enter meiosis within a short prenatal period and the oocytes progress through prophase, becoming arrested in the diplotene stage. After birth, germline cysts break apart as pregranulosa cells enclose individual oocytes to form primordial follicles (Pepling 2006). Thus, in mice, ovarian cyst formation is observed only during embryogenesis. In contrast, in *C. dimerus*, as in other teleosts, clusters of mitotic and meiotic germ cells are frequently found in the germinal epithelium lining the ovigerous lamellae of adult ovaries, indicating that cyst-forming divisions occur throughout much of adult life.

Synchronous development of interconnected germ cell has also been reported in anamniote spermatogenesis (Fawcett *et al.* 1959; Callard 1991; Grier 1993; Schulz *et al.* 2009), as well as in most other animal species, so that cyst-forming division represents a highly conserved type of proliferation which is part of the gametogenic pathway in both sexes (Pepling *et al.* 1999; Saito *et al.* 2007). However, the terminology referring to the 'cysts' and/or 'germline cysts' in the testis and in the ovary of fishes is not uniform and invites confusion. A recent review on fish spermatogenesis states that within the testicular lobules, 'cytoplasmic extensions of Sertoli cells form cysts that envelope a single, clonally and hence synchronously developing group of germ cells deriving from a single spermatogonium' (Schulz *et al.* 2009; page 391) That is, the spermatogenic cyst is formed by Sertoli cells surrounding a group of interconnected germ cells (in other words, each cyst contains a germline cyst). Under this definition, fish are characterized by having a cystic type of spermatogenesis (Callard 1991; Grier 1993; Schulz *et al.* 2009), as opposed to the non-cystic spermatogenesis of amniotes, in which Sertoli cells support at the same time different developmental stages of germ cells, that is cells belonging to different clones (Schulz *et al.* 2009). Then, the amniote testis presents germline cysts (interconnected germ cells) but not cysts. In contrast, the term cyst, i.d., germline cyst, as applied to the female gonad (both in fishes and in other animals) refers to a group of synchronously developing germ cells originated from a single founder cell (Pepling *et al.* 1999) and does not include the surrounding prefollicle/pregranulosa cells. In the female fish, the cyst breaks apart as prefollicle cells individually enclose each oocyte; in the male, the cyst disappears as it opens into the lobular lumen during spermiation.

Germinal epithelium and folliculogenesis after formation of the ovarian lumen

In most teleosts, the ovarian lumen is characteristically formed by the fusion of somatic cell outgrowths that enclose part of the coelom and form a lumen that is internally lined by the former coelomic epithelium. This condition is referred to as ovaries of the cystovarian type (Hoar 1969; Dodd 1977). Mature oocytes are ovulated through this epithelial lining and accumulate within the lumen of the ovary rather than in the

coelomic cavity, as occurs in other vertebrates (Wallace and Selman 1990). Notably, formation of the ovarian lumen in *C. dimerus* occurs in an antero-posterior direction. Coincidently, signs of a cephalo-caudal gradient of gonadal sex differentiation have been verified in other teleosts (Parmentier and Timmermans 1985; Strüssmann and Ito 2005). In the recently differentiated ovaries of *C. dimerus*, the tissue lining the ovarian lumen at the side opposite to the germinal epithelium is formed by a double layer of epithelial cells with connective tissue elements in between. Occasionally, germ cells migrate from the germinal epithelium along the recently formed laminar tissue.

An overview of the characteristics that define the ovarian germinal epithelium in teleosts has been provided by Grier (2000) for *Centropomus undecimalis*. The germinal epithelium in the developing ovary of *C. dimerus* is composed of somatic cells, the epithelial and derived prefollicle cells, and germ cells, either oogonia or early prophase oocytes. It satisfies the criteria that define an epithelium: it borders a body cavity (initially the coelomic cavity and subsequently the ovarian lumen, derived from the former), epithelial cells are adjoined laterally by tight junctions and desmosomes, it is avascular, and it is supported by a basement membrane that separates it from the interstitial compartment. Histologically, a basement membrane always separates the stroma from the germinal epithelium and its derivatives, the ovarian follicles, in the adult ovary. Here, we provide evidence that separation of tissue compartments by the basement membrane is readily apparent from an early stage of gonad development. The significance of the basement membrane in forming this separation is that it is a conserved feature throughout vertebrate evolution (Parenti and Grier 2004). Once the ovarian lumen is formed in the developing ovary of *C. dimerus*, early-stage germ cells are found within inpocketings of the germinal epithelium, where they form cell nests. Within the cell nests, single oogonia, cysts of oogonia and cysts of early prophase oocytes may co-occur. Also, recently formed follicles containing early diplotene oocytes can be observed, as they protrude into the stroma. In *O. latipes*, these regions of the germinal epithelium have been called germinal cradles (Nakamura *et al.* 2010). Within them, nested germ cells at early stages of oogenesis are immersed in a matrix of *sox9b* expressing somatic cells, which correspond to the herein designated prefollicle cells. Germinal cradles lay between external epithelial cells and the basement membrane subtending the germinal epithelium, as observed in *C. dimerus*.

The process of folliculogenesis that we describe here shares characteristics with those reported for other teleosts, both in the adult ovary (Quagio-Grassiotto *et al.* 2011) and during ovarian morphogenesis (Mazzoni *et al.* 2010). When cystic oocytes reach the pachytene step within cell nests of the germinal epithelium, they become separated from each other by cytoplasmic processes of prefollicle cells. As folliculogenesis progresses, the oocyte and associated prefollicle cells protrude into the stroma. Oocyte primary growth is initiated prior to the completion of folliculogenesis. Finally, a diplotene oocyte enveloped by a layer of follicle cells pinches off from the germinal epithelium as a follicle enclosed by a basement membrane derived from that of the germinal epithelium. Prefollicle cells have been reported to synthesize the basement membrane that surrounds the ovarian follicle during this process (Le Menn *et al.* 2007; Lubzens *et al.* 2010). As the follicle arises, stromal cells become organized and give rise to the theca around the basement membrane; thus, the follicle complex is formed. As defined in this study, an ovarian follicle consists of a single oocyte that is surrounded by follicle cells and is separated from the stroma by a basement membrane. This definition is consistent with a follicle being wholly derived from the germinal epithelium (Grier 2000, 2012). The term follicle complex has been introduced to describe the teleost follicle, its basement membrane, and the surrounding layer of thecal cells and blood capillaries (Grier 2000). Accumulating evidence in teleosts indicates that, once formed and throughout its development, the follicle complex remains attached to the germinal epithelium by a region of shared basement membrane (Grier *et al.* 2005, 2007; Mazzoni *et al.* 2010; Quagio-Grassiotto *et al.* 2011; Grier 2012). The point of attachment has been proposed to be the site where the oocyte is released upon ovulation (Grier *et al.* 2009; Quagio-Grassiotto *et al.* 2011; Grier 2012), a region that is both homologous and analogous to that referred to as 'point of rupture' of the mature follicle in the frog's ovary (Rugh 1951).

In the atypical ovaries of the seahorse, *Hippocampus erectus*, two stem cell compartments referred to as germinal ridges are the source of ovarian follicles (Selman *et al.* 1991). The syngnathid germinal ridge has been described as an outpocketing of the luminal epithelium, which contains oogonia, early meiotic oocytes and prefollicle cells. Cells of the germinal ridge and the luminal epithelium lie within a common ovarian compartment that is defined by a continuous underlying basement membrane. Then, the luminal epithelium in the ovary of the female seahorse may be considered a particular kind of germinal epithelium in which oogonia and early prophase oocytes are restricted to two specific sites, that is, the germinal ridges, in contrast to the random organization of most teleosts in which early-stage germ cells are scattered within many inpockets along the luminal epithelium lining the ovigerous lamellae. However, it should be noted that Begovac and Wallace (1988) reported oogonia within the luminal epithelium itself in the ovary of another syngnathid fish, the pipefish, *Syngnathus scovelli*. The particular architecture of the syngnathid ovary in which early germ cells reside within a pocketing of the luminal epithelium may be present in the undifferentiated gonad and recently differentiated ovary of teleosts, as depicted in Fig. 7A,B. The difference would be that early-stage germ cells and their supporting cells initially reside within an inpocket of the coelomic (not luminal) epithelium (as the ovarian lumen has not been formed yet). Later, after the ovarian

lumen develops, germ cells occur within many inpockets of the luminal epithelium (not just two as in the seahorse) (Fig. 7D).

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