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Ovarian Structure and Oogenesis of the Oviparous Goodeids *Crenichthys baileyi* (Gilbert, 1893) and *Empetrichthys latos* Miller, 1948 (Teleostei, Cyprinodontiformes)

Mari Carmen Uribe,[1]* Harry J. Grier,[2,3] and Lynne R. Parenti[2]

[1]*Laboratorio de Biología de la Reproducción Animal, Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 México, D.F. México*
[2]*Division of Fishes, Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, District of Columbia 20013-7012*
[3]*Florida Fish and Wildlife Research Institute, SE, St. Petersburg, Florida 33701-5020*

ABSTRACT The cyprinodontiform family Goodeidae comprises two biogeographically disjunct subfamilies: the viviparous Goodeinae endemic to the Mexican Plateau, and the oviparous Empetrichthyinae, known only from relict taxa in Nevada and California. Ovarian characteristics of two oviparous species of goodeid, *Crenichthys baileyi* and *Empetrichthys latos*, studied using museum collections, are compared with those of viviparous species of goodeids. Both subfamilies have a single, cystovarian ovary. The ovary in the viviparous Goodeinae has an internal septum that divides the ovarian lumen into two compartments, and it may possess oogonia. There is no ovarian septum in the oviparous *C. baileyi* and *E. latos*. Oogenesis is similar in both subfamilies with regard to the proliferation of oogonia, initiation of meiosis, primary growth and development of an oocyte during secondary growth in which fluid yolk progressively fuses into a single globule. Notably, eggs of *C. baileyi* and *E. latos* are approximately double the size of those of the viviparous Goodeinae in which embryos develop inside the ovarian lumen and are nourished, in part, by nutrients transferred from the maternal tissues, a mode of embryo development called matrotrophy. Egg envelopes of the two subfamilies differ in that those of *C. baileyi* and *E. latos* have a relatively thick zona pellucida, attachment fibrils or filaments that develop between the follicle cells during oogenesis, and a micropyle observed only in *E. latos*. In contrast, viviparous goodeid eggs have a relatively thin zona pellucida, but lack adhesive fibrils, and a micropyle was not observed. These reproductive characters are compared with those of species of the eastern North American *Fundulus*, a representative oviparous cyprinodontiform. One newly recognized shared, derived character, a single, median ovoid ovary with no obvious external evidence of fusion, supports monophyly of the Goodeidae. Differences among the goodeid subfamilies and *Fundulus* are interpreted relative to the oviparous versus viviparous modes of reproduction. J. Morphol. 273:371–387, 2012.

KEY WORDS: oogenesis; zona pellucida; micropyle; delle; viviparity; killifish; Atherinomorpha

INTRODUCTION

The viviparous goodeid fishes have long been celebrated in fish reproductive biology for their unique trophotaeniae: short or elongate, somewhat elaborate extensions from the hindgut in embryos and neonates that function in nutrition and respiration (e.g., Turner, 1933, 1937, 1940; Mendoza, 1937, 1940, 1943, 1965; Hubbs and Turner, 1939; Wourms, 1981, 2005; Wourms et al., 1988). Ovarian structure and oogenesis have been described in comparable detail (Turner, 1933, 1947; Mendoza, 1940, 1943; Wourms, 1981, 2005; Wourms et al., 1988; Schindler and Hamlett, 1993; Uribe et al., 2004, 2005, 2009). Anatomical variation in the ovary and the trophotaeniae formed the basis of an early systematic classification (Hubbs and Turner, 1939). Today, the viviparous goodeids comprise 17 genera with 41 species, all endemic to the Altiplano of Central Mexico (Domínguez-Domínguez et al., 2005). Their unique form of viviparity and extensive diversification in freshwater habitats restricted to the Mexican Plateau has generated interest in conservation of the native freshwater fish biota of Mexico: two, possibly three, species are extinct and nearly all others are under some risk (Domínguez-Domínguez et al., 2005).

*Correspondence to: Mari Carmen Uribe, Departamento de Biología Comparada, Facultad de Ciencias, Circuito Exterior, Ciudad Universitaria, Universidad Nacional Autónoma de México, Insurgentes Sur 3000, Delegación Coyoacán, 04510 México, D.F. México. E-mail: uribearanzabal@yahoo.com.mx

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Fig. 1. **A**: *Crenichthys baileyi*, adult female, USNM 391727; **B**: *Empetrichthys latos*, adult female, USNM 391728. Bar = 1 cm.

The relationships of viviparous goodeids to other cyprinodontiforms were unspecified until Parenti (1981) hypothesized that the closest living relatives of the viviparous goodeids are two relictual, oviparous freshwater killifish genera that live in southern Nevada, *Crenichthys* and *Empetrichthys*. The family Goodeidae, therefore, was expanded to comprise two subfamilies: the viviparous Goodeinae of the Mexican Plateau and the oviparous Empetrichthyinae, comprising the two Nevada genera and a fossil species from California. Both groups share derived characters of the oral jaws and the anal fin (Parenti, 1981). The first 6 or 7 anal fin rays of male viviparous goodeids are shortened and unbranched and separated from the rest of the fin. In all goodeids, oviparous and viviparous, the first 2 to 7 middle anal radials are fused to the proximal radials. The sister-group relationship of the Goodeinae and Empetricthyinae was corroborated in subsequent morphological (e.g., Costa, 1998) and molecular (Grant and Riddle, 1995; Webb et al., 2004) analyses.

The Empetrichthyinae comprises 10 recent species or subspecies: *Crenichthys baileyi*, with five subspecies *C. b. baileyi*, *C. b. albivallis*, *C. b. grandis*, *C. b. moapae*, *C. b. thermophilus*, and *C. nevadae*; *Empetrichthys merriami* and *Empetrichthys latos*, with three subspecies *E. l. latos*, *E. l. pahrump*, and *E. l. concavus* (Soltz and Naiman, 1978; Williams and Wilde, 1981). They lack pelvic fins and share a unique form of the first epibranchial (Parenti, 1981: Fig. 47b; Fig. 1). A third species, *Empetrichthys erdsi*, is known only as a fossil from the Los Angeles basin, California (Uyeno and Miller, 1963). They were once somewhat more broadly distributed throughout springs, pools, and streams of the Great Basin and Mojave deserts of Nevada and southern California (Gilbert, 1893) and, like their Mexican sister-group, today suffer intense habitat deterioration and degradation (Minckley and Marsh, 2009). *E. merriami* and all but one subspecies of *E. latos* are extinct (Minckley and Deacon, 1968; Miller et al., 1989). The extant *E. l. latos*, endemic to a single spring in Manse Ranch from which it has been extirpated, persists in refugia outside its native Pahrump Valley (Soltz and Naiman, 1978; Minckley and Marsh, 2009). *C. baileyi* is listed as threatened (Minckley and Marsh, 2009).

In contrast to our extensive knowledge of viviparous goodeid reproduction, we are aware of only the briefest details of reproduction of empetrichthyines (Hubbs, 1932; Hubbs and Miller, 1948; Miller, 1948; Kopec, 1949). Wild-caught *C. baileyi* laid 10–17 eggs, one at a time, during spawning events. Eggs measured 1.9 mm in diameter, and eggs had adhesive fibrils or filaments by which they are attached to vegetation (Kopec, 1949). Fertilized eggs hatched in 5–7 days in the laboratory (Kopec, 1949). Reproduction is asynchronous; females spawn at least twice per year (Minckley and Marsh, 2009).

There are no studies of *Empetrichthys* or *Crenichthys* ovarian morphology and oogenesis. Taxa of both genera are extinct, endangered, or threatened. We were fortunate to have the opportunity to examine archival collections made in the mid-1960s and stored at Arizona State University (ASU). In addition to comparing the ovaries and oogenesis in both oviparous and viviparous goodeids, we include data on the oviparous cyprinodontiform, *Fundulus*, for outgroup comparison. Here, we describe the morphology of the ovary and the process of oogenesis of *C. baileyi* and *E. latos*.

MATERIALS AND METHODS

C. baileyi, National Museum of Natural History, Smithsonian Institution (USNM) 391727 (ex. ASU 5169), collected from Crystal Springs, Lincoln County, Nevada, 28 November 1965, by Wilson and collecting party three adult females (42–48 mm standard length (SL); Fig. 1A). *E. latos*, USNM 391728 (ex. ASU 12137), collected from Manche Ranch spring, Nye County, Nevada, 2 July 1967, by Hubbs and collecting party one adult female (41 mm SL; Fig. 1B). Specimens were fixed in 10% formalin and subsequently stored in 70% ethanol at ASU and then transferred to the USNM. In November 2009, the ovaries were excised through a mid-lateral incision in the abdomen. Whole ovaries were embedded in glycol methacrylate (JB-4 plastic, Polysciences®), sectioned at 6 μm and stained with hematoxylin and eosin (H-E), and periodic acid-Schiff/metanil yellow (PAS/MY; Quintero-Hunter et al., 1991). Follicle measurements were recorded from the histological preparations using a calibrated ocular micrometer. The oocyte development classification of Grier et al. (2009) was used to define stages of oogenesis. In that classification, oocyte development is divided into six stages, each with a two-letter, upper case abbreviation: oogonial proliferation (OP), chromatin-nucleolus (CN), primary growth (previtellogenesis) (PG), secondary growth (vitellogenesis) (SG), oocyte maturation (OM), and ovulation. The stages are divided into steps, identified with a lower case abbreviation, which are designed to identify the morphological changes in oocytes during growth and maturation.

RESULTS

Histological elements of the ovary are germ cells (oogonia and oocytes in different stages of develop-

ment) and somatic tissues (somatic cells of the germinal epithelium, vascularized stroma, smooth muscle, and peritoneum). Even though the ovaries processed in this study were fixed in the mid-1960s, the histological preparations revealed good preservation of ovarian tissues. The histological characters of the ovaries and of oogenesis of *C. baileyi* and *E. latos* are comparable; our descriptions of the ovary and of oocyte development pertain to both species. The illustrations of *C. baileyi* histology are presented in Figures 2–5, and that of *E. latos* in Figures 6–9; images of both species are combined in Figure 10.

Ovarian Components

C. baileyi and *E. latos* have a single ovary, ovoid in shape, and longitudinally suspended from the dorsal wall by the mesovarium. The ovary is of cystovarian type, containing a central lumen. Irregular lamellae or folds project from the ovarian wall into the lumen. Lamellae are composed of stroma that exhibits a diversity of follicular stages of development (Figs. 2A,B and 6A,B). The surface of the lamellae is lined by the germinal epithelium. The germinal epithelium, as are all epithelia, is separated from the stroma by a basement membrane. The germinal epithelium contains oogonia and early oocytes that are scattered among somatic epithelial cells (Figs. 2C and 6C) or as extensions of the germinal epithelium that form cell nests. Each follicle is encompassed by a basement membrane which separates it from the stroma.

Oogenesis and Folliculogenesis

Oogenesis and folliculogenesis in both *C. baileyi* and *E. latos* begins when oogonia proliferate and enter meiosis to become oocytes. During folliculogenesis, early oocytes in cell nests are enclosed progressively by somatic epithelial cells that become prefollicle cells. The prefollicle cells, in turn, become follicle cells when they, and the oocyte, form a follicle (Figs. 2D,E and 6C). They are then completely surrounded by a basement membrane. Subsequently, a thin, vascularized theca, derived from the stroma, develops around the basement membrane. The structure formed by the follicle (oocyte and follicle cells), and surrounding elements (basement membrane and theca) form the ovarian follicle complex. Subsequently, oocytes undergo morphological and physiological changes throughout the stages of oogenesis defined as in the classification of Grier et al. (2009).

Oogonia Proliferation Stage

Oogonia are scattered among somatic epithelial cells in the germinal epithelium (Figs. 2C–E and 6C). Oogonia are spherical cells with an approximate diameter of 8–10 μm. The ooplasm is hyaline. The nucleus is spherical and contains a single nucleolus and fine granular chromatin. The oogonia initiate meiosis, entering the CN stage of development; then they are oocytes.

Chromatin-Nucleolus Stage

The oocyte is in CN stage (Fig. 2D,E) when meiosis begins. It advances through leptotene, zygotene, pachytene, and early diplotene of prophase I of meiosis when, gradually, the chromosomes condense and are seen as thin threads. During early diplotene, lampbrush chromosomes develop and the oocyte begins to grow, reaching 10–12 μm in diameter. The ooplasm of early diplotene oocytes is hyaline, similar to that seen in the oogonia, as folliculogenesis proceeds. The prefollicle cells completely enclose the oocyte, beginning to define the developing follicular structure.

Primary Growth Stage or Previtellogenesis

The PG stage comprises: one nucleolus step (PGon), multiple nucleoli step (PGmn), perinucleolar step (PGpn), and oil droplets—cortical alveoli step (PGod).

One nucleolus step (PGon). The size of the oocytes increases to ~40 μm. The nucleus of the oocyte, now called a germinal vesicle, is spherical and enlarges in volume, reaching ~15 μm in diameter. The germinal vesicle contains chromosomes that are arrested in the diplotene step as characteristic lampbrush chromosomes, and a single, spherical nucleolus (Figs. 2D and 6D). The ooplasm becomes progressively more basophilic as ribonucleic acids are synthesized in the germinal vesicle by the nucleolus and are transported to the ooplasm. The follicle cells form a squamous cell layer (2–3 μm high), completely surrounding the oocyte.

Multiple nucleoli step (PGmn). The oocytes attain a diameter of ~80 μm. The oocyte's germinal vesicle now contains multiple nucleoli (Figs. 2D–F and 6D) that are randomly distributed. Basophilia of the ooplasm is intense, as in the previous step. The Balbiani bodies, irregular structures of the ooplasm adjacent to the germinal vesicle, are seen (Figs. 2E,F and 6D). The follicle cells form a squamous cell layer, similar to those in the previous step. The basement membrane and the vascularized theca completely surround the follicle.

Perinucleolar step (PGpn). The oocyte attains a diameter of ~150 μm. The germinal vesicle is spherical and large, about 60 μm in diameter. The multiple nucleoli become oriented around the inner membrane of the germinal vesicle (Figs. 3A and 6C). The nucleoli are spherical and have varying diameters (2–4 μm). Balbiani bodies are around the periphery of the germinal vesicle (Fig. 3A). There is a slight decrease in ooplasmic

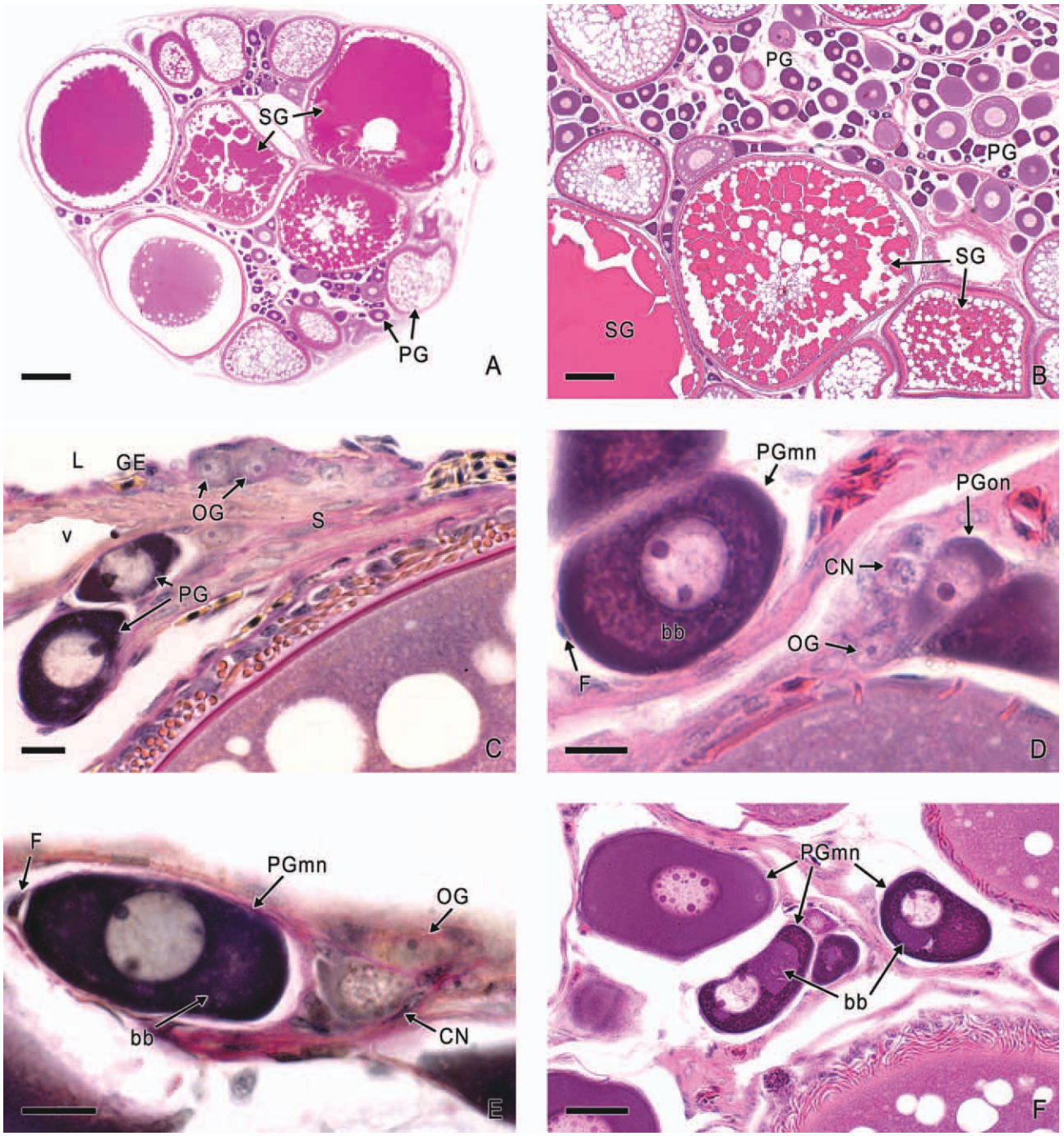

Fig. 2. Ovary of *Crenichthys baileyi*. **A** and **B**: Panoramic views of the single ovary containing abundant follicles in different stages of development; the progressive enlargement in diameter of growing follicles is evident. The presence or absence of yolk and its amount differentiates the stages of oogenesis. Primary growth stage (PG), secondary growth stage (SG). A: H-E. Bar = 500 μm. B: H-E. Bar = 200 μm. **C**: The germinal epithelium (GE) borders the ovarian lumen (L). There are oogonia (OG) that are clearly differentiated from somatic epithelial cells because of their round shape, light ooplasm, and spherical nucleus with one nucleolus. Oocytes during primary growth (PG) are seen. Blood vessels (v) are seen in the stroma (S) that is subjacent to the germinal epithelium. PAS/MY-H. Bar = 10 μm. **D** and **E**: Germinal epithelium with an oogonium, (OG). Oocytes during chromatin nucleolus stage (CN), defined by the nucleus with chromosomes as fine filaments during early prophase I of meiosis. Oocytes in primary growth with basophilic ooplasm during the one nucleolus step (PGon), and multiple nucleoli step (PGmn) with a clear increase in diameter. Balbiani bodies (bb) are seen. Follicle cells (F) surround the oocytes. D: H-E. Bar = 12 μm, E: PAS/MY-H. Bar = 12 μm. **F**: Oocytes in multiple nucleoli step of primary growth (PGmn), presenting progressive increase in the number of nucleoli. Balbiani bodies (bb) are seen around the germinal vesicle. H-E. Bar = 50 μm.

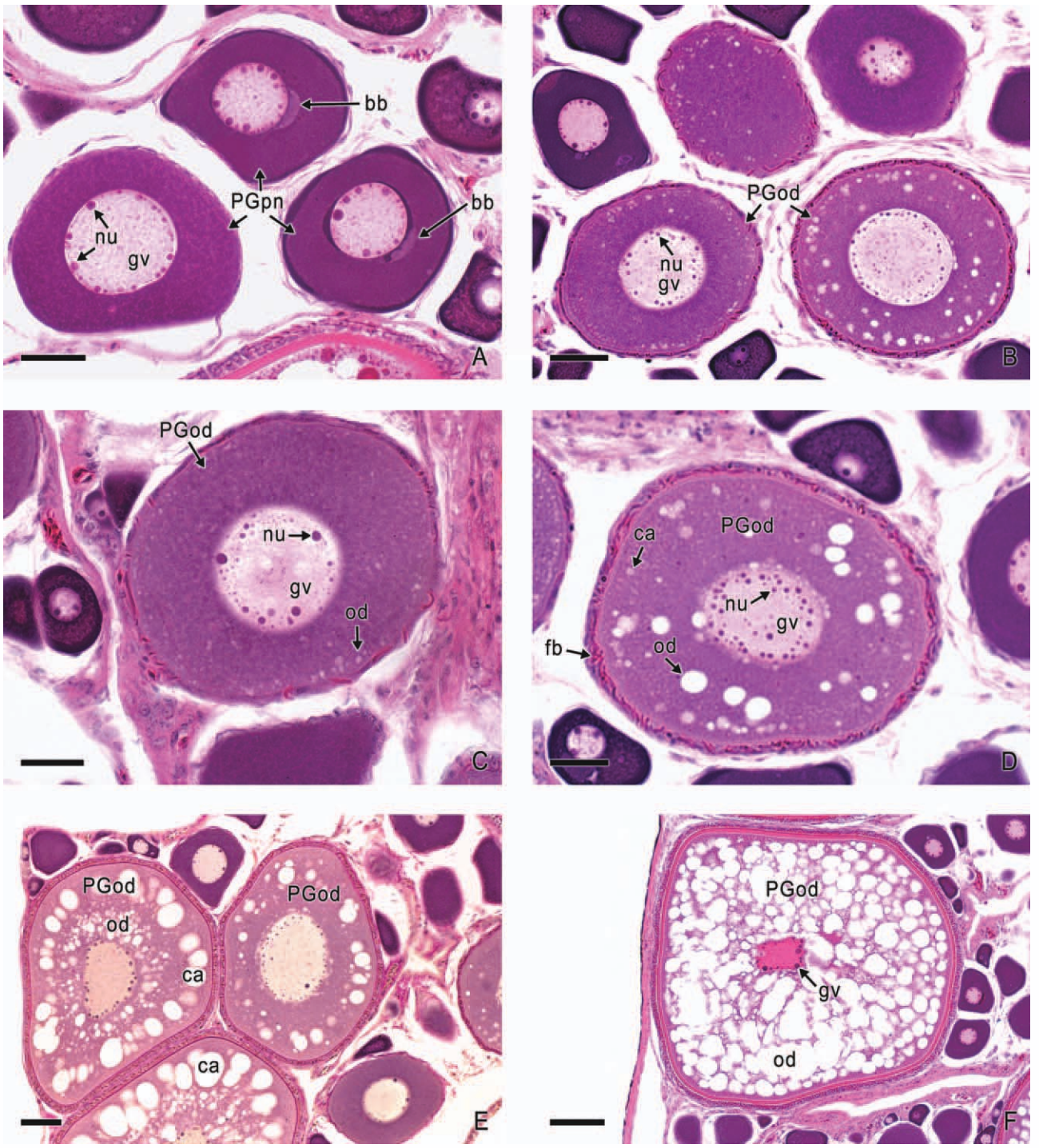

Fig. 3. Oocytes during the primary growth stage in ovary of *Crenichthys baileyi*. **A**: Oocytes in the perinucleolar step (PGpn) of primary growth. The germinal vesicle is spherical and large. Most of the large nucleoli (nu) become oriented around the inner membrane of the germinal vesicle (gv). Balbiani bodies (bb) are seen. H-E. Bar = 50 μm. **B**: Oocytes during the oil droplets step (PGod) of primary growth. The germinal vesicle (gv) contains numerous nucleoli (nu). H-E. Bar = 50 μm. **C**: Oocyte during the oil droplets step (PGod). The germinal vesicle (gv) is situated at the center of the oocyte. The ooplasm contains scarce oil droplets (od). H-E. Bar = 50 μm. **D**: Oocyte during the oil droplets step (PGod). The germinal vesicle (gv) presents numerous nucleoli (nu). There is a progressive increase in the number of oil droplets (od) and cortical alveoli (ca). Peripheral acidophilic fibrils (fb) are distributed between the follicle cells. H-E. Bar = 50 μm. **E**: Oocyte during the oil droplets step (PGod). Oil droplets (od) and cortical alveoli (ca) are more numerous. PAS/MY-H. Bar = 50 μm. **F**: At the end of oil droplets step (PGod), the germinal vesicle (gv) has irregular folds. The oil droplets (od) are located throughout the ooplasm. H-E. Bar = 100 μm.

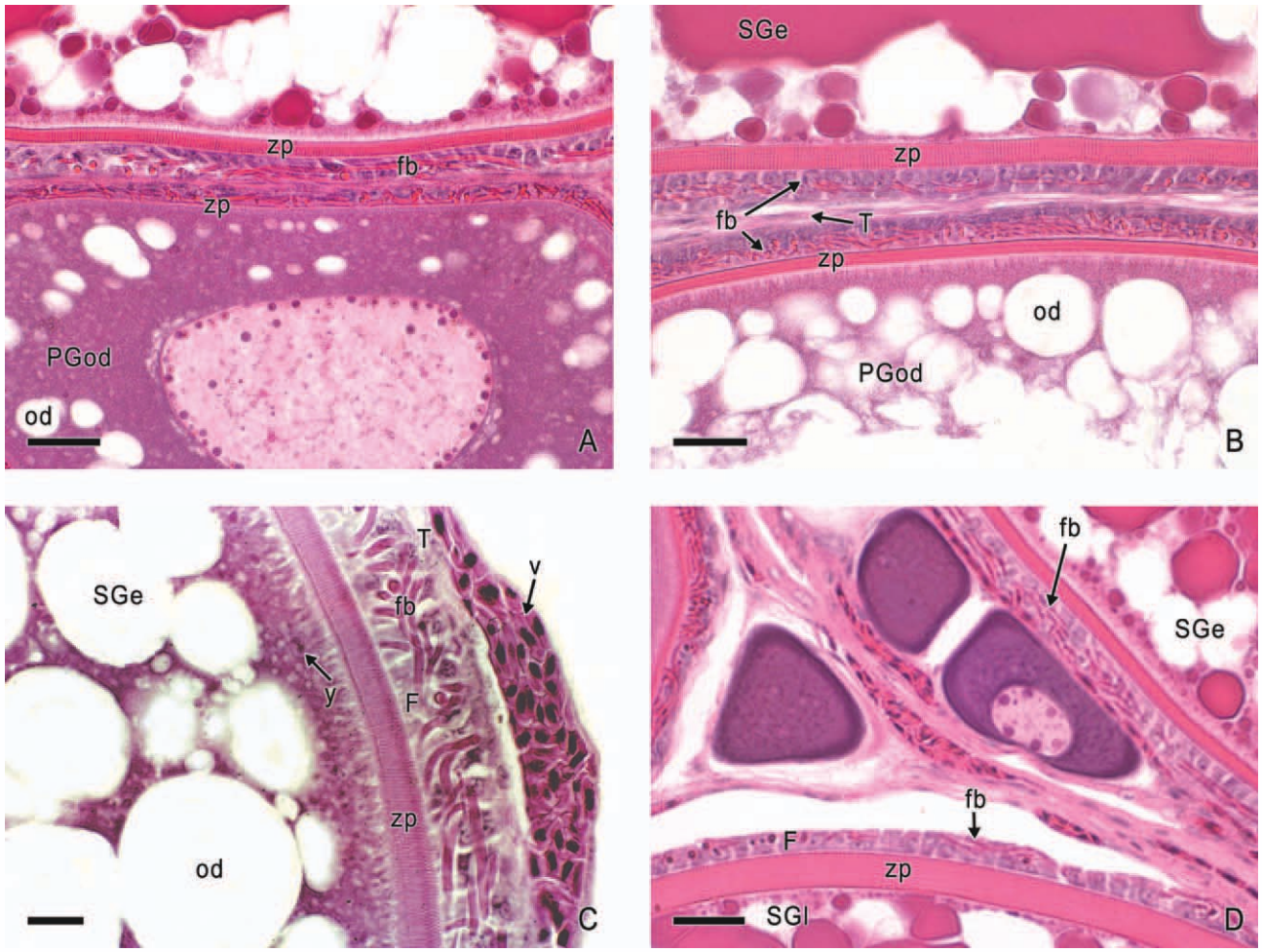

Fig. 4. Zona pellucida, follicle cells and theca of the follicle complex of *Crenichthys baileyi*. **A–D**: There is an increase in the thickness of the zona pellucida (zp) between the oil droplets step (PGod) of primary growth and the early (SGe) and late steps (SGl) of secondary growth. Oil droplets (od) and early deposition of yolk globules (y) is seen. Columnar follicle cells (F) with acidophilic fibrils (fb) are distributed between them. The theca (T) contains blood vessels (v). A: H-E. Bar = 25 μm. B: H-E. Bar = 25 μm. C: PAS/MY-H. Bar = 10 μm. D: H-E. Bar = 25 μm.

basophilia. The follicle cells remain squamous, as in the preceding two steps. The vascularized theca is similar to those in the previous step.

Oil droplets and cortical alveoli step (PGod). The oocytes reach ~550 μm in diameter. Multiple nucleoli are positioned around the periphery of the germinal vesicle. Oil droplets and cortical alveoli appear around the germinal vesicle, later, they are dispersed throughout the ooplasm. Spherical oil droplets (Figs. 3B–E and 7A–C) increase progressively in number and size; some of them attain 50 μm in diameter. Cortical alveoli are spherical and vary in diameter (Figs. 3D,E and 7A–C). At first appearance, some of them are 2 μm in diameter, but later they may reach ~25 μm. Then, there is a massive accumulation of oil droplets throughout the ooplasm (Figs. 3F and 7D,E). The zona pellucida is well defined (Figs. 4A,B and 7F) as a homogeneous line, densely stained, 2–3 μm thick. At the end of this step, the zona pellucida (Figs. 4B and 7F) reaches 10–12 μm thick and is clearly striated. The follicle cell layer (Fig. 7F) is thicker than in the perinucleolar step; its cells become cuboidal and attain a mean height of 5 μm. Acidophilic fibrils are located between the follicle cells in various positions (Figs. 4A,B and 7F), they are seen in circular, longitudinal, or spiral arrangements. The vascularized theca (Figs. 4B and 7F) is similar to that seen in the previous step. At the end of this step, the germinal vesicle has irregular folds (Figs. 3F and 7D,E).

Secondary Growth Stage or Vitellogenesis

The SG stage comprises: early secondary growth step (SGe), late secondary growth step (SGl), and full-grown oocyte step (SGfg).

Early secondary growth step (SGe). The oocytes grow to a great extent through the active deposition of yolk, attaining a diameter of 1,200 μm.

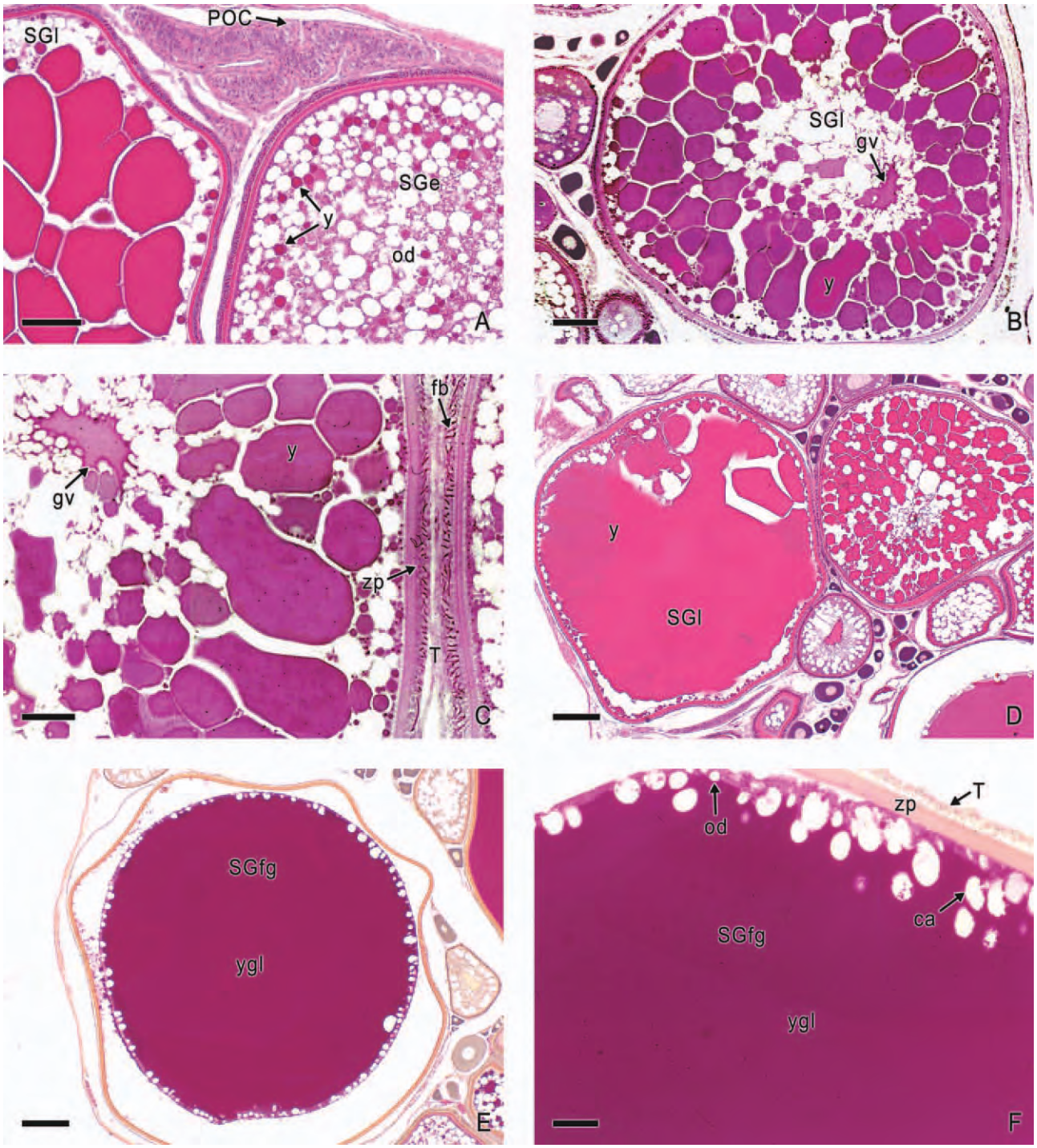

Fig. 5. Oocytes during the secondary growth stage in ovary of *Crenichthys baileyi*. **A**: Oocyte during early secondary growth step (SGe) when the early deposition of yolk globules (y) appears in the ooplasm. The yolk globules are among abundant oil droplets (od). An oocyte during late secondary growth step (SGl) and a postovulatory follicle complex (POC) are also seen. H-E. Bar = 100 μm. **B** and **C**: Two magnifications of an oocyte during late secondary growth step (SGl) when yolk globules (y) progressively fuse. The germinal vesicle (gv) is eccentric, and its periphery has irregular folds. The zona pellucida (zp), follicle cells with fibrils (fb) between them, and theca (T) surround the follicle. B: H-E. Bar = 100 μm. C: H-E. Bar = 50 μm. **D**: Oocyte during late secondary growth step (SGl) presenting clear increase of the yolk (y) fusion. H-E. Bar = 250 μm. **E** and **F**: The oocyte has reached its maximum size during the full-grown oocyte step (SGfg). The smaller yolk globules become one large yolk globule (ygl) that occupies most of the oocyte volume. Oil droplets (od) and cortical alveoli (ca) are at the periphery of the ooplasm. The zona pellucida (zp) and theca (T) surround the follicle. E: PAS/MY-H. Bar = 250 μm. F: PAS/MY-H. Bar = 50 μm.

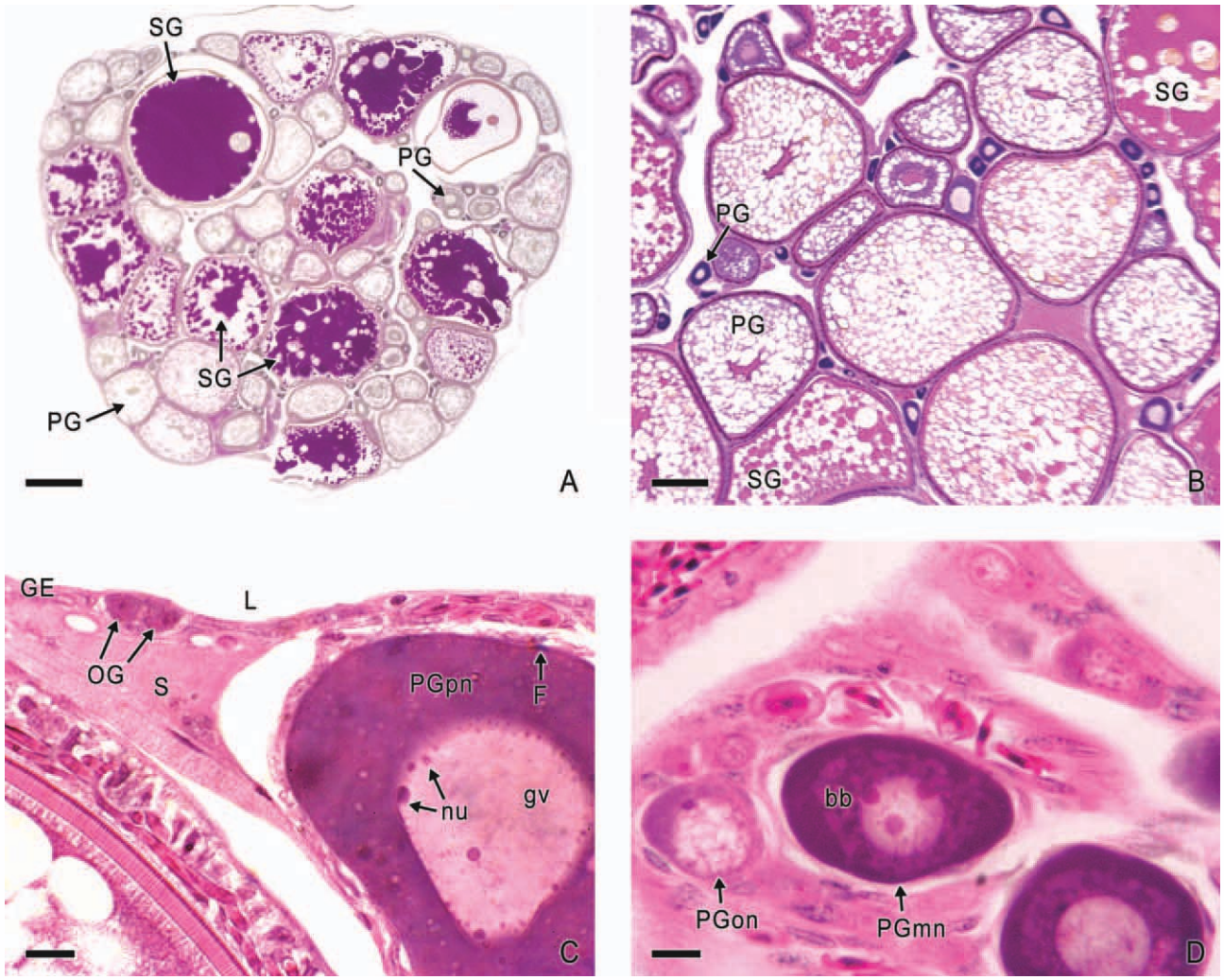

Fig. 6. Ovary of *Empetrichthys latos*. **A** and **B**: Panoramic views of a single ovary containing abundant follicles in different stages of development, the progressive enlargement in diameter of the follicles is evident as they grow. The presence or absence of yolk and its amount differentiates the stages of oogenesis. Primary growth stage (PG), secondary growth stage (SG). A: PAS/MY-H. Bar = 500 μm. B: H-E. Bar = 200 μm. **C**: The germinal epithelium (GE) borders the ovarian lumen (L). Within it, oogonia (OG) possess spherical nuclei and one nucleolus. An oocyte during the perinucleolar step (PGpn) of primary growth is also seen, the germinal vesicle (gv) is large and spherical, with the nucleoli (nu) oriented peripherally. Squamous follicle cells (F) are seen. The stroma (S) is subjacent to the germinal epithelium. H-E. Bar = 10 μm. **D**: Oocytes in primary growth during the steps of one nucleolus (PGon), and multiple nucleoli (PGmn). The increase of ooplasmic basophilia is evident in multiple nucleoli (PGmn) by intense stained with hematoxylin. Balbiani bodies (bb) are seen near the germinal vesicle. H-E. Bar = 10 μm.

Folding of the germinal vesicle continuous at its periphery (Fig. 8A) that began in the previous step. The oil droplets are abundant and located throughout the ooplasm (Figs. 5A and 8A). Early spherical, deeply acidophilic yolk vesicles, with a diameter of 2–5 μm, begin to accumulate at the oocyte periphery (Figs. 4C and 5A). The yolk vesicles progressively increase in number and size. Throughout this step, the yolk vesicles begin to fuse to form ovoid or irregular yolk globules (Fig. 8A). The zona pellucida is 15–16 μm thick and is a well-differentiated, striated layer between the oocyte and the follicle cells (Fig. 4B,C). The follicle cells remain as a single layer throughout SG, but become columnar, attaining 12–14 μm in height (Fig. 4C). The acidophilic fibrils (Fig. 4B–D), located between the follicular cells, progressively increase in number. The vascularized theca (Fig. 4C) is similar to that described earlier.

Late secondary growth step (SGl). The germinal vesicle is eccentric and deeply folded along its periphery (Figs. 5B,C and 8B). The fusion of the yolk globules advances to form large regions of fluid yolk within the ooplasm (Figs. 5B–D and 8B–F). The zona pellucida attains 20 μm and appears clearly striated (Figs. 4D, 5C, and 8D–F). The micropyle was observed in the zona pellucida of oocytes of *E. latos* (Fig. 9A,B). The micropyle is a

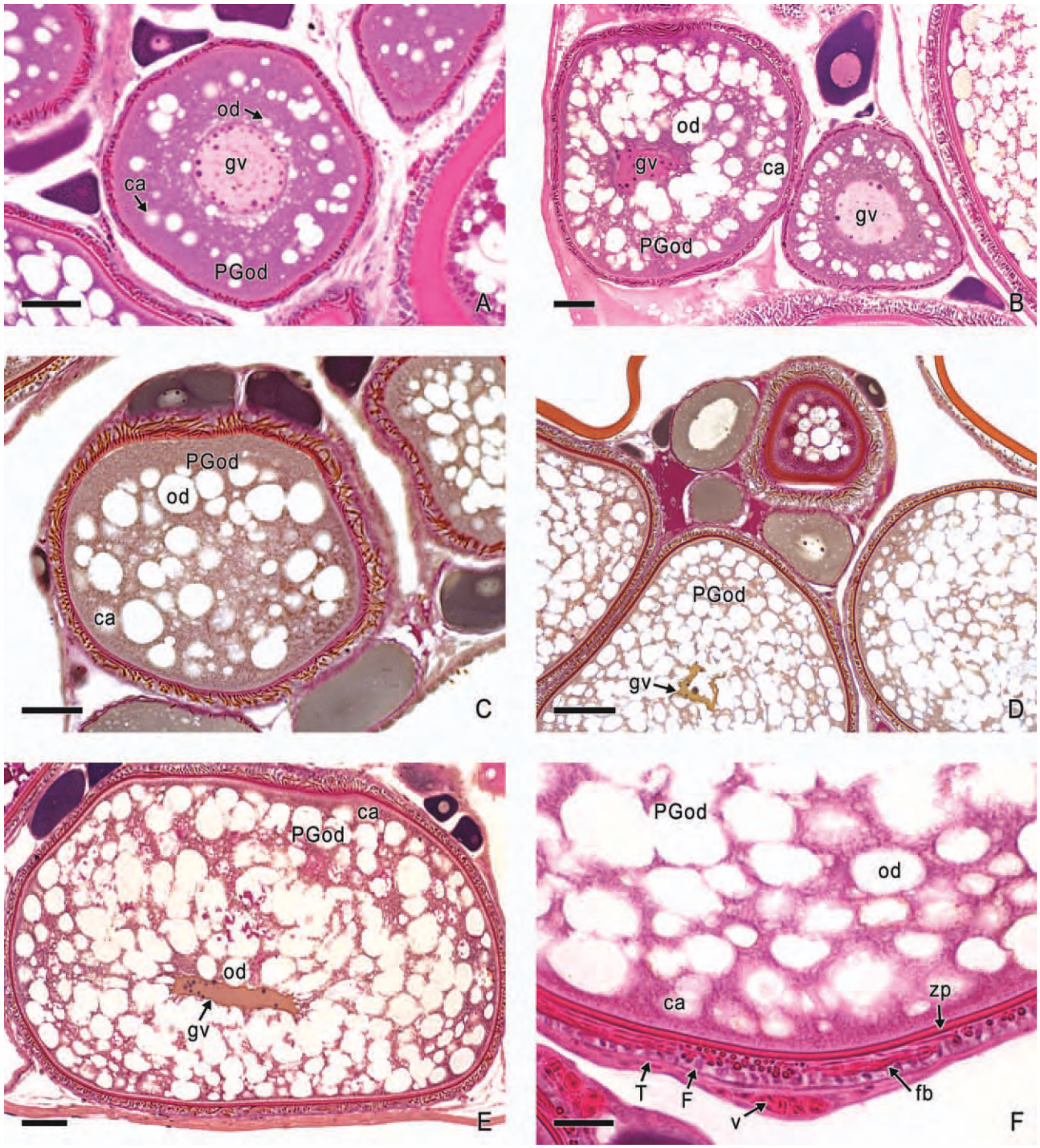

Fig. 7. Oocytes during the primary growth stage in ovary of *Empetrichthys latos*. **A**–**D**: Oocytes during oil droplets step (PGod) of primary growth clearly illustrate the increase of the number of oil droplets (od) and cortical alveoli (ca). The germinal vesicle (gv) is changing from spherical and centrally located to irregular in shape and eccentric. A: H-E. Bar = 50 μm. B: H-E. Bar = 50 μm. C: PAS/MY-H. Bar = 50 μm. D: PAS/MY-H. Bar = 100 μm. **E** and **F**: At the end of oil droplets step (PGod), the germinal vesicle (gv) is eccentric, and its periphery has irregular folds. The oil droplets (od) are located throughout the ooplasm. The zona pellucida (zp) is well defined. Peripheral acidophilic fibrils (fb) are distributed between the follicle cells (F). The theca (T) contains blood vessels (v). E: H-E. Bar = 50 μm. F: H-E. Bar = 25 μm.

well-defined, channel-shaped opening. At its exterior edge, close to the follicle cell layer, the micropyle is ~4 μm in diameter. It is slightly smaller at the interior edge of the zona pellucida, near the ooplasm, where its diameter is 3 μm (Fig. 9B). The sides of the micropyle are undulated,

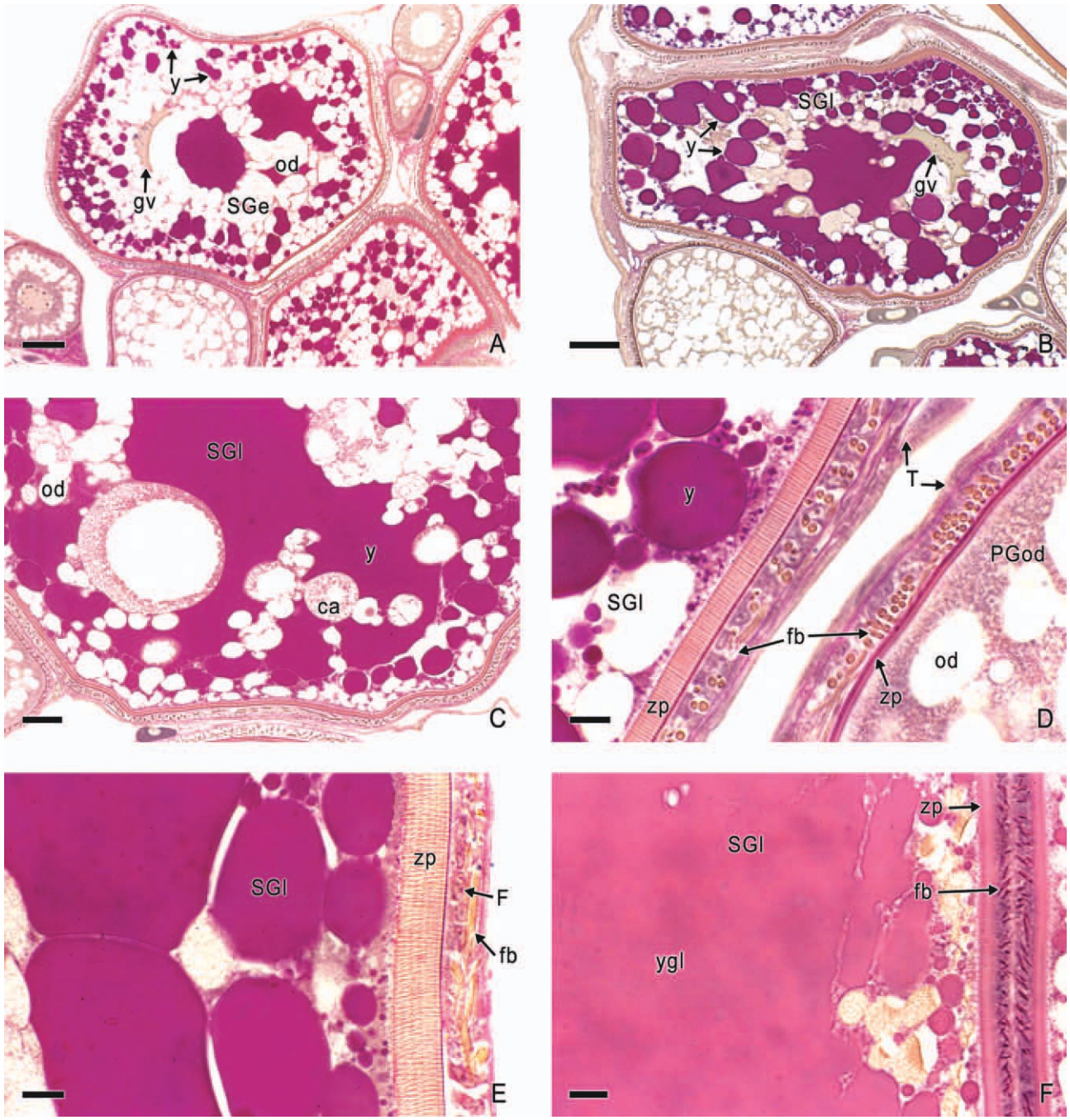

Fig. 8. Oocytes during the secondary growth stage in ovary of *Empetrichthys latos*. **A**: Oocytes during early secondary growth step (SGe) when the early deposition of yolk globules (y) appears in the ooplasm. The yolk globules are located among abundant oil droplets (od). The germinal vesicle (gv) is eccentric, and its periphery has irregular folds. PAS/MY-H. Bar = 100 μm. **B–F**: Oocytes during the late secondary growth step (SGl) when yolk globules (y) progressively fuse. Oil droplets and cortical alveoli (ca) are seen. The germinal vesicle (gv) remains eccentric. The yolk globules become one large yolk globule (ygl) that occupies most of the oocyte volume. The zona pellucida (zp), follicle cells (F), and theca (T) surround the follicle. The zona pellucida (zp) thickens between the oil droplets step (PGod) of primary growth, early secondary growth (SGe), and late secondary growth steps (SGl). Fibrils (fb) distributed between the follicle cells are observed. B: PAS/MY-H. Bar = 100 μm. C: PAS/MY-H. Bar = 50 μm. D: PAS/MY-H. Bar = 10 μm. E: PAS/MY-H. Bar = 20 μm. H-E. F: Bar = 10 μm.

following the striated structure of the zona pellucida. The layer of follicle cells remains single, but decreases slightly in height, to 10–12 μm, compared with that observed in the previous step. The acidophilic fibrils are in the same position between the follicle cells (Figs. 5C and 8D–F). The theca (Figs. 5C and 8D) is similar to that described previously.

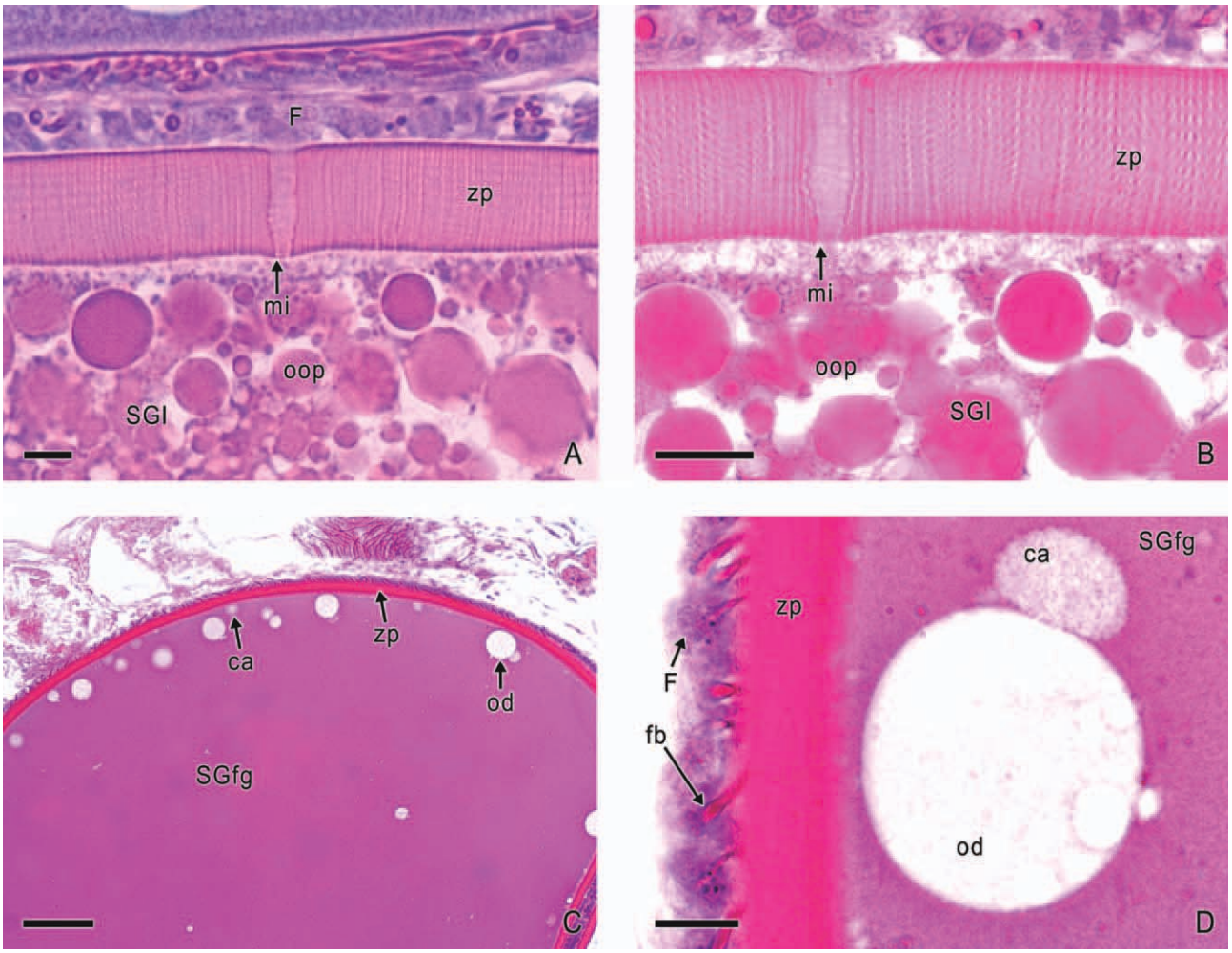

Fig. 9. Oocytes during the secondary growth stage in ovary of *Empetrichthys latos*. **A** and **B**: Micropyle in an oocyte in late secondary growth step (SGl). The micropyle (mi) opening in the zona pellucida (zp) is slightly smaller at the interior edge near the ooplasm (oop). Follicle cells (F) surround the zona pellucida. A: H-E. Bar = 10 μm. B: H-E. Bar = 12 μm. **C** and **D**: The oocyte reaches its maximum size during the full-grown oocyte step (SGfg). The yolk globules fuse and become one large yolk globule that occupies most of the oocyte volume. Oil droplets (od) and cortical alveoli (ca) are at the periphery of the ooplasm. The zona pellucida (zp) increases thickness as oogenesis advances, and the striation is not seen. Acidophilic fibrils (fb) distributed between the follicle cells (F) are observed. C: H-E. Bar = 100 μm. D: H-E. Bar = 12 μm.

Full-grown oocyte step (SGfg). The oocytes reach their maximum diameter, attaining 1.8–2 mm, in both species. The yolk globules are fused completely and form a large fluid globule of yolk (Figs. 5E,F and 9C). Oil droplets and cortical alveoli encircle the periphery of the ooplasm (Figs. 5E,F and 9C,D). The zona pellucida is 18–20 μm thick, and there are no evident striations (Fig. 9D). The follicular cells form a layer of 8–10 μm, slightly smaller than those seen in the previous step. The acidophilic fibrils are seen (Fig. 9D). The theca is similar to that described previously. Some full-grown oocytes were shrunken and the zona pellucida, follicle cells, and theca were separated from the oocyte by a space (Fig. 5E). Such shrinkage was likely due to formalin or Bouin's fixation or the embedding medium.

Throughout oogenesis, the germinal vesicle undergoes discrete morphological changes (Fig. 10A–D). Initially, during OP and CN stages, and PGon, PGmn (Fig. 10A), and PGpn steps, the germinal vesicle is spherical or ovoid in shape. Then, during the PGod step (Fig. 10B), it becomes slightly irregular in shape. Small and large nucleoli are seen. Finally, in the SG stage (Fig. 10C,D), it has an elongate, irregular shape, and its envelope becomes deeply folded. Small and large nucleoli are also seen.

Postovulatory follicle complexes (Fig. 5A) in both species indicate that the specimens were collected during the spawning season.

DISCUSSION

The histological analysis of the ovary and oogenesis in *C. baileyi* and *E. latos*, species so rare in the wild, permitted this description and documen-

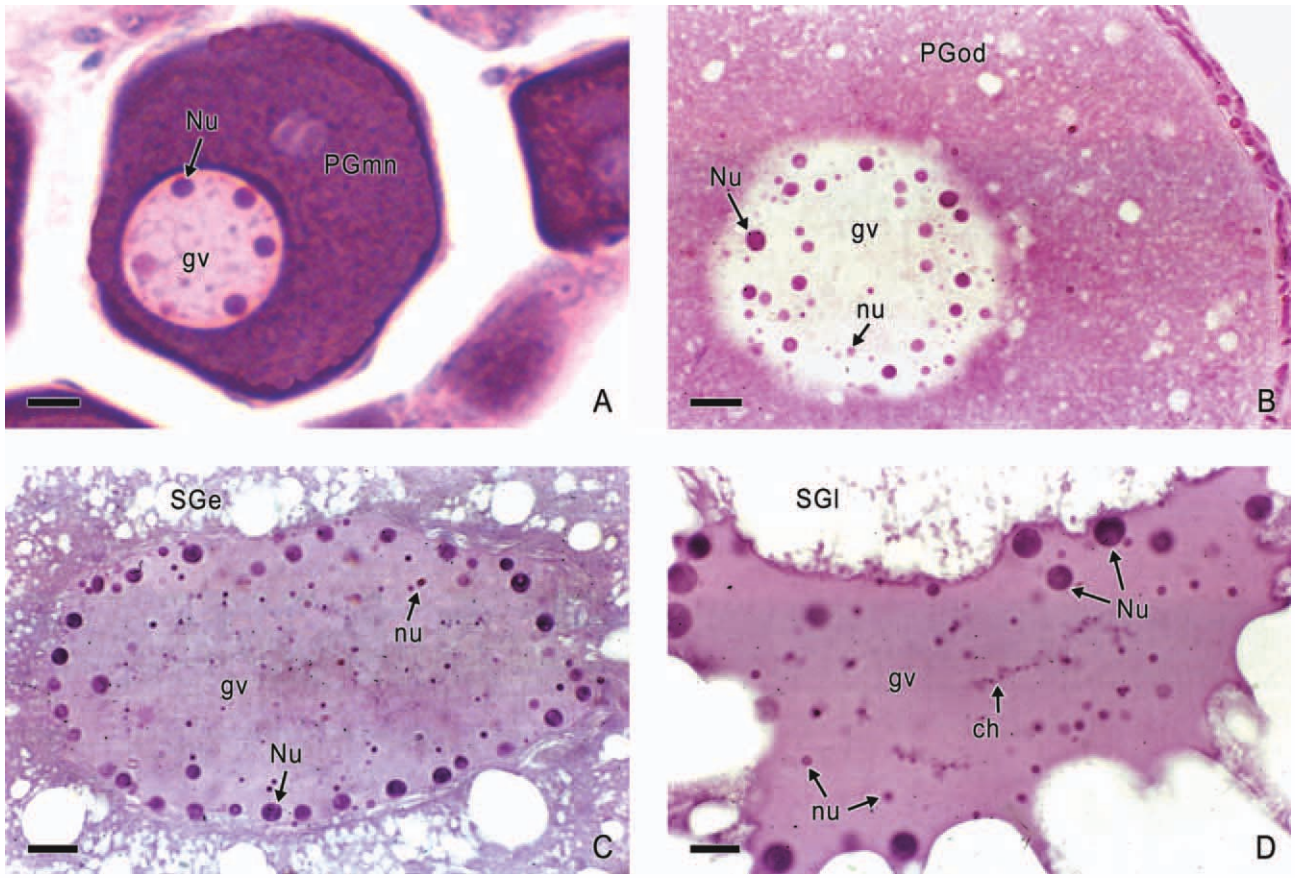

Fig. 10. Sequence of morphological changes of the germinal vesicle during oogenesis in *Crenichthys baileyi* (A,C) and *Empetrichthys latos* (B,D). **A** and **B**: In oocytes in primary growth stage, during multiple nucleoli step (PGmn) and oil droplets step (PGod), having a spherical shape. Large nucleoli (Nu) and small nucleoli (nu) are seen in the germinal vesicle. A: H-E. Bar = 10 μm. B: H-E. Bar = 10 μm. **C** and **D**: In oocytes in secondary growth stage, during early secondary growth step (SGe) and late secondary growth step (SGl), having the germinal vesicle deeply folded and large peripheral nucleoli. There are also numerous small nucleoli (nu) that are less peripheral than the large ones (Nu). Lampbrush chromosomes (ch) are also seen. C: H-E. Bar = 10 μm. D: H-E. Bar = 10 μm.

tation of essential aspects of their reproduction. Here, we summarize and compare the earlier suite of reproductive characters among the two subfamilies of goodeid and the oviparous *Fundulus*, first describing the characters that all three atherinomorph taxa share (Parenti, 2005), and then those characters that vary and allow us to make statements about relationship. Character states that vary are also tabulated (Table 1).

Shared Characters

Germinal epithelium. Activity of the germinal epithelium underlies the seasonality of the female reproductive cycle. The germinal epithelium lines the ovarian lumen and is the location of the oogonia, which divide by mitosis, proliferate and develop into the next generation of germ cells during the reproductive cycle (Grier, 2000). Oogonia divide mitotically and become oocytes when they enter meiosis. Mitosis of oogonia is the first stage (OP) in the classification of oogenesis in teleosts presented by Grier et al. (2009), and is documented here in the oogenesis of *C. baileyi* and *E. latos*. A germinal epithelium has also been documented in *Fundulus* and several species of viviparous goodeids (Parenti and Grier, 2004; Grier et al., 2005).

Follicle cells. Follicle cells remain as a single layer throughout oogenesis. Changes in follicle cells during follicle development in *C. baileyi* and *E. latos* range from being a squamous layer during PG to cuboidal or columnar cells during SG, with a reduction to cuboidal or squamous again in full-grown follicles. These changes are similar to those described in viviparous goodeids (Mendoza, 1965; Uribe et al., 2004, 2005, 2009). Likewise, similar morphological changes were described in the oviparous cyprinodontiforms *Fundulus heteroclitus* by Matthews (1938) and *Cynolebias melanotaenia* and *Cynolebias ladigesi* by Wourms (1976). The increase in follicle cell size is related to the active process of vitellogenesis during SG. The follicle cells are involved in the synthesis of a diversity of

TABLE 1. Comparison of reproductive characters among female Fundulus, Empetrichthys, Crenichthys, and viviparous Goodeids (for citations, see text)

Reproductive characters	*Fundulus*	*Empetrichthys*	*Crenichthys*	Viviparous Goodeids
Reproductive mode	Oviparous	Oviparous	Oviparous	Viviparous
Oocyte diameter	1.7–1.9 mm	1.8–2 mm	1.8–2 mm	0.5–1 mm
Zona pellucida thickness	12 μm	18–20 μm	18–20 μm	2–3 μm
Egg fibrils or filaments	Present	Present	Present	Absent
Sperm passageway to the egg	Micropyle	Micropyle	Not seen	Delle
Ovarian structure	Single, bilobed anteriorly, no septum	Single, ovoid, no septum	Single, ovoid, no septum	Single, ovoid, with septum

proteins and lipids during the growth of the oocytes and, consequently, they become smaller once vitellogenesis is complete (Guraya, 1986).

Folliculogenesis. Folliculogenesis in *C. baileyi* and *E. latos* is complete when a layer of prefollicle cells, derived from the somatic epithelial cells of the germinal epithelium, entirely enclose each oocyte to form follicle cells that are surrounded by a basement membrane (Grier, 2000; Grier et al., 2005, 2009). The follicle consists of an oocyte and surrounding follicle cells (Grier, 2000). The follicle is enclosed by a basement membrane and theca to form the ovarian follicle complex. Development of the follicle is included in the second stage (CN) of the classification of oogenesis (Grier et al., 2009), and continues into PG. Descriptions of the elements of folliculogenesis are similar in many teleosts (for reviews see, Dodd and Sumpter, 1984; Selman and Wallace, 1989). The stages and steps developed during oogenesis in *C. baileyi* and *E. latos* are similar to those reported for other atherinomorphs, including *Fundulus* and viviparous goodeids.

Cystovarian condition. The ovary of *C. baileyi* and *E. latos* is cystovarian; it has a central lumen of coelomic origin. The ovarian wall possesses ovigerous folds or lamellae that are lined by germinal epithelium and project into the lumen (Dodd and Sumpter, 1984; Grier, 2000). The lamellae contain ovarian follicles in different stages of development and the mature oocytes are ovulated into the ovarian cavity, features documented in several other cyprinodontiform taxa, including *Fundulus* (Matthews, 1938; Brummett et al., 1982; Dodd and Sumpter, 1984; Guraya, 1986; Grier, 2000; Parenti and Grier, 2004) and viviparous goodeids (Grier et al., 2005, 2009).

Primary growth stage. The initial steps of PG, common not only to teleosts but also other vertebrates that develop yolked oocytes, are characterized by basophilia of the ooplasm. This indicates a period of intense RNA synthesis coupled with ribosome production to support development of the oocyte and the embryo (Wallace and Selman, 1990; Patiño and Sullivan, 2002; Grier et al., 2009). Initially, during PG, Balbiani bodies are seen around the periphery of the germinal vesicle; they subsequently migrate to the oocyte periphery and disperse. Balbiani bodies have been observed in previtellogenic oocytes in viviparous goodeids (Uribe et al., 2005, 2009) as well as other viviparous and oviparous species (Droller and Roth, 1966; Azevedo, 1984; Guraya, 1986; Kobayashi and Iwamatsu, 2000).

During PG, cortical alveoli and oil droplets develop gradually in relation to the increase in oocyte diameter (Selman et al., 1988). This process is similar not just in atherinomorphs but numerous other teleosts (for reviews see, Dodd and Sumpter, 1984; Selman and Wallace, 1989; Tyler and Sumpter, 1996; Grier et al., 2009). The initial oil droplets are located around the germinal vesicle in both *C. baileyi* and *E. latos*, agreeing with the description in *F. heteroclitus* by Selman et al. (1988) and Selman and Wallace (1989), viviparous goodeids and numerous other teleost species (for reviews see Guraya, 1986; Grier et al., 2009).

The similar morphology of cortical alveoli and oil droplets observed in *C. baileyi* and *E. latos*, during the early oil droplets step of primary growth (PGod) make their differentiation difficult. This observation agrees with those of Selman and Wallace (1989) and Tyler and Sumpter (1996), who both indicated that oil droplets are difficult to distinguish from cortical alveoli because the contents of both are leached out because of inadequate fixation in the histological process. In addition, the empetrichthyine specimens examined here were preserved in alcohol for nearly half a century, which may have compromised the integrity of the oil droplets and cortical alveoli.

Secondary growth stage. Oocytes of *C. baileyi* and *E. latos* enlarge greatly to attain their maximum diameter during SG due to the synthesis and accumulation of yolk. Yolk is the fundamental material stored in the ooplasm during SG, as in other teleosts (Guraya, 1986; Wallace and Selman, 1990). Yolk is used for the nutrition and metabolic activities during embryonic development. Yolk proteins are derived from vitellogenin, the hepatic yolk precursor. Vitellogenin is a glycolipophosphoprotein transported by blood vessels from the liver to the ovarian follicle and is taken up by developing oocytes. Vitellogenin enters the oocytes by binding to specific receptors on the oolemma. Then, it is internalized by endocytosis. Once in the

ooplasm, vitellogenin is divided into smaller molecular weight polypeptides: the yolk proteins lipovitellin, phosvitin, and β′-component. The yolk proteins are then packed into yolk globules, which are stored in developing oocytes, and used as an energy source for embryonic development (Patiño and Sullivan, 2002; LaFleur et al., 2005; Raldúa et al., 2006; Grier et al., 2009). During SG, yolk fuses in *C. baileyi* and *E. latos*, similar to that described in the species of viviparous goodeids (Mendoza, 1943; Wourms, 1981; Schindler and Hamlett, 1993; Uribe et al., 2004, 2005, 2009). Yolk is fluid also in *F. heteroclitus* (see Matthews, 1938; Selman and Wallace, 1983; Selman et al., 1986) and in other atherinomorphs (Wallace and Selman, 1980, 1981; Guraya, 1986; Selman et al., 1986; Selman and Wallace, 1989; Parenti and Grier, 2004; Jalabert, 2005; Grier et al., 2009). This may result from a deep reorganization of the lipoprotein yolk involving the action of proteolytic enzymes, as suggested by Jalabert (2005). Fluid yolk is one diagnostic character of atherinomorphs that supports their monophyly (Parenti and Grier, 2004).

Characters that Vary

Ovarian structure. Most teleosts have paired ovaries suspended via a mesovarium from the dorsal wall of the coelom. A single, median ovary is typical of viviparous and some oviparous teleosts (Grier et al., 2009). All viviparous goodeids and *C. baileyi* and *E. latos* have a single, median ovoid ovary with no obvious external traces of fusion. A single ovary, resulting from fusion of both ovaries during embryological development, has been documented in viviparous goodeids by numerous authors, including Hubbs and Turner (1939), Turner, (1947), Amoroso (1960), Mendoza (1965), Wourms (1981), Dodd and Sumpter (1984), Wourms et al. (1988), and Schindler and Hamlett (1993). The ovary of *F. heteroclitus* was described by Matthews (1938, p 70) as unpaired, however, bilobed anteriorly with a mid-ventral groove that separates it superficially into right and left portions.

Ovarian septum. The ovary has an internal septum that divides the ovarian lumen into two compartments in all species of viviparous goodeids (Turner, 1947; Mendoza, 1965; Wourms, 1981; Schindler and Hamlett, 1993; Uribe et al., 2004, 2005, 2009). Gestation occurs in both chambers of the ovarian lumen (Uribe et al., 2005). There is no ovarian septum in the oviparous *C. baileyi* and *E. latos*, or in *F. heteroclitus* (see Matthews, 1938).

Oocyte size. Egg size is a direct indicator of fish development (Elinson, 1989). There is a distinct difference in egg size between species of the two subfamilies of goodeids. The maximum oocyte diameter in both *C. baileyi* and *E. latos* is 1.8–2 mm. The oocyte diameter of the oviparous *F. heteroclitus* is 1.7–1.9 mm (Selman and Wallace, 1983; Selman et al., 1986), similar to that observed here, and in Kopec (1949), for *C. baileyi* and *E. latos*. In contrast, the maximum diameter of oocytes of viviparous goodeids is much smaller: 1.0 mm in *Ateniobius toweri* (Turner, 1940), 0.8 mm in *Goodea atripinnis*, *Characodon lateralis* (Turner, 1940; Uribe et al., 2005), and *Xenotoca* (=*Characodon*) *eiseni* (Mendoza, 1965), 0.7 mm in *Ilyodon whitei* (Uribe et al., 2004, 2005), and 0.5 mm in *Skiffia* (=*Goodea*) *bilineata* (Turner, 1933), for example. The reduced diameter of full-grown oocytes in viviparous goodeids is related to the intraluminal gestation in which embryos obtain nutrients from the mother, a process known as matrotrophy. These additional nutrients are contained in the histotrophe secreted by the ovarian epithelium into the ovarian lumen (Turner, 1937, 1947; Amoroso, 1960; Mendoza, 1972; Wourms, 1981, 2005; Schindler and De Vries, 1987). During gestation, transfer of nutrients from the mother to the embryo occurs through different means. The histotrophe may be absorbed by the trophotaeniae, extensions of the hindgut of the embryo into the ovarian lumen that are lined with absorptive epithelium (Turner, 1937; Wourms, 1981, 2005; Wourms et al., 1988). Schindler and Greven (1992) documented endocytosis of proteins from the histotrophe by the trophotenial absortive cells in the viviparous goodeid *Ameca splendens*. Also, embryos may be oophagous (they eat and digest eggs from the ovarian lumen) or adelphophagous (they eat and digest other embryos; Greven and Großherr, 1992).

Cortical alveoli. Cortical alveoli are observed in Goodeinae and Empetrichthyinae species, but in the latter, cortical alveoli are larger, sometimes attaining 50 μm in diameter. Large cortical alveoli have also been documented in *F. heteroclitus* (see Anderson, 1968; Selman et al., 1988; Wallace and Selman, 1990).

Fluid yolk. Yolk is fluid throughout the oocyte during SG in *C. baileyi* and *E. latos* as described also in viviparous goodeids and in all other atherinomorph fishes as far as known (Parenti and Grier, 2004; Parenti, 2005). In viviparous goodeids, fluid yolk comprises most of the ooplasm (Wourms et al., 1981; Uribe et al., 2005). In contrast, in *C. baileyi* and *E. latos*, as in *F. heteroclitus*, the oocyte also has oil droplets at its periphery (Matthews, 1938; Wallace and Selman, 1990).

Zona pellucida. The zona pellucida in full-grown oocytes of *C. baileyi* and *E. latos* is 18–20 μm thick, comparable with the thickness recorded in *F. heteroclitus* by Brummett (1966) and Kuchnow and Scott (1977). The increase in the number of microvilli, which gives the zona pellucida its striated appearance during oocyte growth, amplifies the surface of interchange between oocyte and follicle cells. During oogenesis, the germ cells and follicle cells remain connected by a variety of

adhering junctional complexes. Intercellular communication by gap junctions between the oocyte and follicle cells during OM in *F. heteroclitus* was suggested by Cerdà et al. (1993) to have an important functional role in the transfer of various substances. They further propose that such communication is necessary for the maintenance of meiotic arrest and hydration that occurs by the translocation from follicle cells to maturing oocyte of K^+, the primary osmotic effector for oocyte hydration. During OM, oocyte microvilli may decrease in number mitigating the striated appearance of the zona pellucida (Cerdà et al., 1999). Similar changes in the zona pellucida surrounding oocytes in late oogenesis were reported in the oviparous seahorse, *Hippocampus erectus* and pipefish, *Syngnathus fuscus* by Anderson (1967), and croaker, *Micropogonias undulatus* by York et al. (1993), and also in a viviparous halfbeak, *Dermogenys pusillus* by Flegler (1977). Reduction of the striated appearance of the zona pellucida in *F. heteroclitus* was described by Kuchnow and Scott (1977). In addition, Dumont and Brummett (1980) suggested that the changes in the structure of the zona pellucida of *F. heteroclitus* may be related to changes in the oocyte volume through hydration during maturation. In contrast to the thick zona pellucida of oviparous species, a thin zona pellucida is formed by the follicle of viviparous species (Flegler, 1977; Uribe et al., 2005, 2009). The zona pellucida of oocytes, during late SG step, in viviparous goodeids, such as *I. whitei* and *G. atripinnis*, reaches 2–3 μm in thickness (Uribe et al., 2009).

Micropyle. A single micropyle, an opening in the zona pellucida at the animal pole through which the sperm enters the oocyte, is a synapomorphy of the Actinopterygii, the ray-finned fishes (Bartsch and Britz, 1997). A micropyle in the zona pellucida of *F. heteroclitus* was documented by Kuchnow and Scott (1977) and Dumont and Brummett (1980), and in *Fundulus grandis* by Grier et al. (2009). A micropyle was observed in *E. latos* (Fig. 9A,B) and is surrounded by a striated zona pellucida indicating that it forms before OM, during late SG. A micropyle was not seen in *C. baileyi*.

Although widely reported in oviparous species, a micropyle has never been documented in a viviparous species (Grier et al., 2009; Uribe et al., 2009). In viviparous goodeids, the sperm enters the oocyte through a "delle" (named by Stuhlmann, 1887): a thin, funnel-like channel that connects the ovarian lumen with the oocyte. A micropyle is a structure in the zona pellucida through which a sperm pass to reach the oocyte surface. A delle is a passageway from the ovarian lumen to the ovum formed by cells of the germinal epithelium. Sperm reach the oocyte through a small opening in the epithelium at the base of the delle (see Bailey, 1933, pp 207–208) and presumably pass through a micropyle to reach the oocyte surface; fertilization is intrafollicular. The presence of a micropyle has yet to be demonstrated in viviparous goodeids. Homology of a micropyle and an opening at the base of the delle is not endorsed here and is a topic for further study.

Fibrils. Elongate, adhesive fibrils or filaments between the follicular cells on the surface of developing oocytes characterize both *C. baileyi* and *E. latos*. Fibrils are formed by the follicle cells (Anderson, 1967; Dumont and Brummett, 1980). Fibrils or filaments over all or a portion of the egg surface are characteristic of many other oviparous cyprinodontiform species such as *F. heteroclitus*, has been documented extensively (e.g., Kemp and Allen, 1956; Shanklin, 1959; Brummett, 1966; Kuchnow and Scott, 1977; Dumont and Brummett, 1980; Brummett and Dumont, 1981). Fertilized eggs adhere to vegetation via fibrils. Fibrils may also protect the egg as they trap debris and obscure the embryo from view (Brummett, 1966). They may also help to prevent water loss during low tide when the embryo may be exposed (Dumont and Brummett, 1980). Eggs of all viviparous species lack fibrils on the surface of the zona pellucida (Uribe et al., 2005, 2009; Parenti et al., 2010). Absence of fibrils is interpreted as an evolutionary loss of a structure that is no longer needed in fishes in which development is intraovarian.

CONCLUSIONS

Goodeid embryos develop in two different environments: the exterior in the oviparous species, and within the ovarian lumen in viviparous species. Size and external surface features of the egg reflect the environment in which the embryo develops (Guraya, 1986). Eggs of the oviparous *Crenichthys* and *Empetrichthys* are relatively large and covered with adhesive fibrils or filaments. They have a thick zona pellucida. A micropyle was seen only in *E. latos*. They share these characters with other oviparous taxa, such as species in the genus *Fundulus* discussed here. Viviparous goodeids have relatively small eggs, no adhesive fibrils, and a delle rather than a micropyle, characters consistent with their reproductive mode. Also, cortical alveoli are large in *Empetrichthys*, *Crenichthys*, and *Fundulus* relative to those in viviparous goodeids.

A single derived reproductive character is shared by all viviparous goodeids and *C. baileyi* and *E. latos*: a single, median ovoid ovary with no obvious external evidence of fusion. This is in contrast to the oviparous *Fundulus* which has a single ovary separated only superficially by a mid-ventral groove into right and left portions (Matthew, 1938). A single, median ovary characterizes all viviparous and some oviparous cyprinodontiforms, as well as other teleosts (Grier et al., 2009). We interpret this character as a synapomorphy of the Goodeidae; however, because the character is so

widespread among teleosts, we hesitate to infer that it represents an intermediate state between oviparity and viviparity. Other reproductive characters, especially those of males, need to be surveyed before we can hypothesize about the stages in evolution of viviparity among goodeids.

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