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Testicular structure and spermatogenesis of the oviparous goodeids *Crenichthys baileyi* (Gilbert, 1893) and *Empetrichthys latos* Miller, 1948 (Teleostei, Cyprinodontiformes)

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Abstract

The cyprinodontiform family Goodeidae comprises some 51 species, including subspecies, of freshwater fishes all of which are at risk or are extinct in the wild. It is classified in two allopatric subfamilies: the Goodeinae, endemic to the Mexican Plateau, and the Empetrichthyinae, known only from relict taxa in Nevada and southern California. The 41 species of goodeins are all viviparous and share a set of well-documented reproductive characters. In contrast, the recent species or subspecies of empetrichthyins are all oviparous and relatively poorly known, yet of critical interest in understanding the evolution of livebearing in the family. We previously described ovarian structure and oogenesis in empetrichthyins using archival museum specimens of females and here extend that study to males. Testicular characters of two species of empetrichthyins, *Crenichthys baileyi*, and *Empetrichthys latos*, are studied and compared directly with those of one species of viviparous goodeid, *Ataeniobius toweri*. The testis is a restricted spermatogonial type in both the Empetrichthyinae and the Goodeinae: spermatogonia are found solely at the distal termini of lobules, a diagnostic character of atherinomorph fishes. Morphology of the differentiation of germinal cells during spermatogenesis is similar in both subfamilies. In the oviparous *C. baileyi* and *E. latos* spermatozoa are free in the deferent ducts. In contrast, the spermatozoa of viviparous goodeids are organized into numerous bundles called spermatozeugmata, a characteristic of most fishes that practice internal fertilization. Differences between the goodeid subfamilies are interpreted relative to the oviparous versus viviparous modes of reproduction. Archival museum specimens are a reliable source of data on reproductive morphology, including histology, and may be the only specimens available of rare or extinct taxa.

KEYWORDS

archival museum specimens, atherinomorph testis, internal/external fertilization, sperm transfer mechanisms

1 | INTRODUCTION

The Mexican Plateau is home to an array of endemic species of freshwater fishes that reflects the region's complex geologic and biogeographic history (Domínguez-Domínguez, Mercado-Silva, & Lyons, 2005; Miller, Minckley, & Norris, 2005; Miller & Smith, 1986). The some 41 species of viviparous goodeids (Goodeinae, Goodeidae, Cyprinodontiformes) are "… the most distinctive element in the central Mexican fish fauna…" (Miller & Smith, 1986, pp. 495). They are distinguished by a set of reproductive morphological features that have long been the subject of intensive study (e.g., Hubbs & Turner, 1939; Turner, 1933, 1937; Mendoza, 1965, 1972; Wourms, 1981, 2005; Schindler & Hamlett, 1993; Uribe, De la Rosa-Cruz, & García-Alarcón, 2005, 2009; Schindler, 2014). Their sister taxon is the subfamily Empetrichthyinae (Grant & Riddle, 1995; Parenti, 1981; Webb et al., 2004) classified in two genera, *Crenichthys* and *Empetrichthys*, known from relict populations in Nevada and southern California (Soltz &

‡Deceased

Naiman, 1978). In contrast to the viviparous goodeins, the empetrichthyins are oviparous. Species-level taxonomy of these two genera is controversial. Williams and Wilde (1981) recognized 10 species or subspecies of *Crenichthys* and *Empetrichthys*, whereas Campbell and Piller (2017) argue that there is little justification for recognition of subspecies and that molecular differentiation among lineages supports recognition of about five valid species, four in *Crenichthys* and one in *Empetrichthys*.

The disjunct distribution of the two subfamilies of goodeids is inferred to be the result of vicariant processes such as disruption of a widespread ancestral range (Parenti, 1981) and plate tectonics (Miller & Smith, 1986). Continuous habitat degradation and intense population pressure threatens all species of goodeids throughout their ranges in Mexico and the United States (Domínguez-Domínguez et al., 2005; Miller, Williams, & Williams, 1989; Minckley & Marsh, 2009). Conservation measures include maintenance of captive populations of viviparous goodeids in the Laboratorio de Biología Acuática, Universidad Michoacana de San Nicolás de Hidalgo in Morelia (Domínguez-Domínguez et al., 2005).

Collection of new specimens in the wild of many goodeid taxa, especially the empetrichthyins, is restricted or impossible due to extinction. We know only the briefest details of reproduction of empetrichthyins: wild-caught *Crenichthys baileyi* laid 10 to 17 eggs, one at a time during spawning events; reproduction is asynchronous and females spawn at least twice per year (Minckley & Marsh, 2009).

Archival museum specimens have proven to be a rich source of data for studies of comparative gonad morphology (e.g., Grier & Parenti, 1994; Grier, Uribe, Lo Nostro, Mims, & Parenti, 2016; Parenti & Grier, 2004; Parenti, Grier, & Uribe, 2015). For many vertebrate taxa, especially rare, endangered, or extinct species, historical museum specimens may be the only study material available. If initial preservation is good, museum specimens may be used for decades after their date of collection to study details of reproductive and other morphology (see esp. Parenti et al., 2015). Further, this ensures that a voucher specimen and the data associated with it are archived for future use, including verification of observations.

We were privileged to be able to examine the histology of the ovaries and oogenesis of archival collections of specimens of female empetrichthyins made in the mid-1960s in Nevada and stored at Arizona State University, and transferred subsequently to the National Museum of Natural History, Smithsonian Institution (Uribe, Grier, & Parenti, 2012). Abundant material of two species was at hand: *C. baileyi* (Gilbert, 1893) and *Empetrichthys latos* Miller, 1948. In that study, we proposed a new, diagnostic character of the family Goodeidae: a single, median, ovoid ovary with no obvious external evidence of fusion of paired ovaries (Uribe et al., 2012). Other oviparous cyprinodontiforms have paired, not fused ovaries. Therefore, oviparous empetrichthyins, with a single, median ovary are more like their viviparous sister taxon than other oviparous fishes. This demonstrates the value of these archival specimens in understanding the evolution of livebearing in the family.

Differences between female goodeins and empetrichthyins were also noted (Uribe et al., 2012: their Table 1). In *C. baileyi* and *E. latos*, eggs are 1.8 to 2 mm in diameter, approximately double the size of those of viviparous goodeins in which embryos are nourished, not only by the yolk stored during oogenesis, but also by nutrients transferred from the maternal tissues during gestation (Schindler, 2014; Uribe et al., 2005; Wourms, Grove, & Lombardi, 1988). The eggs of female *C. baileyi* and *E. latos* also have a relatively thick zona pellucida, 18 to 20 μm, and adhesive filaments that develop between the follicle cells during oogenesis by which expelled ova may attach to vegetation (Kopec, 1949; Uribe et al., 2012). These egg characters are shared with other oviparous cyprinodontiforms. In contrast, eggs of viviparous goodeids have a relatively thin zona pellucida, 2 to 3 μm, and lack adhesive filaments, both considered unnecessary for intraovarian gestation (Uribe et al., 2012). The testicular structure and spermatogenesis of viviparous goodeids have been well-described (Grier, 1981; Grier, Fitzsimons, & Linton, 1978; Meisner, 2005; Uribe et al., 2010; Uribe, Grier, De La Rosa-Cruz, & García-Alarcón, 2009; Uribe, Grier, & Mejía-Roa, 2015). Male goodeins have a restricted spermatogonial lobular testis (sensu Parenti & Grier, 2004). This testis is characterized by longitudinal lobules that run from its periphery to the deferent ducts. The spermatogonia lie exclusively at the periphery of the testis lobules. During spermatogenesis, the germ cells develop synchronously within spermatocysts, the perimeter of which is composed of Sertoli cells. Cysts at later stages of spermatogenesis are located progressively closer to the efferent ducts.

There are no published studies of the male reproductive structures of the oviparous empetrichthyins. Aspects of reproductive biology of these species are unknown, including morphology of the testis and the process of spermatogenesis. Here, we provide complementary data on the testis and spermatogenesis from a study of males of the oviparous goodeids *C. baileyi* and *E. latos* from the same ASU collections that supplied the females. To compare directly the testis structure of *C. baileyi* and *E. latos* with a viviparous goodeid, we include the histology of the testes of the viviparous species *Ataeniobius toweri*.

2 | MATERIALS AND METHODS

2.1 | Fish specimens

Crenichthys baileyi, USNM 391727 (ex. ASU 5169), collected from Crystal Springs, Lincoln County, Nevada, November 28, 1965, by Wilson and collecting party, three adult males (34-43 mm standard length, SL; Figure 1a).

Empetrichthys latos, USNM 391728 (ex. ASU 12137), collected from Manche Ranch spring, Nye County, Nevada, July 2, 1967, by Hubbs and collecting party, one adult male (30 mm SL; Figure 1b).

Specimens of both species were fixed in 10% formalin and subsequently transferred to 75% ethanol for long-term storage.

Ataeniobius toweri. This species of viviparous goodeid is known only from the Río Verde drainage in the state of San Luis Potosí, México (Miller, Minckley, & Norris, 2005). It is considered endangered in nature. Captive populations collected from the La Media Luna, Pánuco basin, San Luis Potosí, are maintained at the Laboratorio de Biología Acuática, Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán (Domínguez-Domínguez et al., 2005) from which we obtained three adult males in May 2010, and three adult males in August 2010.

FIGURE 1 A: *Crenichthys baileyi*, adult male, USNM 391727, Bar = 1 cm; B: *Empetrichthys latos*, adult male, USNM 391728, Bar = 1 cm

2.2 | Histology of testes

Testes were excised through a mid-lateral incision in the abdomen. Whole testes were embedded in glycol methacrylate plastic (JB-4 embedding kit, Polysciences, Inc., Warrington, PA), sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Measurements were recorded from histological slides using a calibrated ocular micrometer.

2.3 | Deposition of materials

Specimens of empetrichthyins, including histological slides, are maintained in the Division of Fishes, National Museum of Natural History (USNM), Smithsonian Institution, Washington, D.C. Additional information on this collection is available from the USNM online catalog at: http://collections.nmnh.si.edu/search/fishes/. Specimens of *Ataeniobius toweri* are in the Laboratorio Biología de la Reproducción Animal, Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad Universitaria, DF, México.

3 | RESULTS

Even though the specimens of the oviparous goodeids used in this study were collected and fixed in formalin in the mid-1960s and stored for decades in 75% ethanol, histological preparations of the testes revealed excellent initial tissue fixation and preservation. The testicular structure and spermatogenesis of both oviparous species, *C. baileyi* and *E. latos*, is comparable in histological sections. The images of testicular histology of *C. baileyi* are presented in Figures 2–4, and those of *E. latos* in Figures 5–7. These six figures provide equivalent histological structures of both species; therefore Figure 2 may be compared with Figure 5, Figure 3 with Figure 6, and Figure 4 with Figure 7. To contrast morphology of these male oviparous goodeids with the viviparous goodeids, the testis of *A. toweri* is illustrated in Figure 8, especially to emphasize the structure of the spermatozeugmata, bundles of spermatozoa, which are not formed in oviparous goodeids.

3.1 | Testicular structure

Testes are paired, elongate organs attached to the dorsal wall of the body by a mesorchium. Histological examination of the testes revealed that they are composed of germinal and interstitial compartments separated by a basement membrane. The germinal compartment consists of two cell types: germ cells and somatic, epithelial Sertoli cells. The interstitial compartment surrounds the lobules, comprised of Leydig cells and connective tissue that is integrated by fibroblasts, collagen fibers, macrophages, lymphocytes, myoid cells, blood vessels, and nerve fibers. Germ cells include all the stages of differentiation of the spermatogenic cells. Spermatogenesis proceeds within a cystic structure in which all germ cells develop in a synchronous clone that is surrounded by Sertoli cells.

The testis is a restricted spermatogonia lobular testis type in which spermatogonia are confined to the testicular periphery, as in *C. baileyi* (Figure 2a) and *E. latos* (Figure 5a). A longitudinal section of the testis, from the testicular periphery to the central deferent duct, reveals that the lobules have an orderly progression of developing germ cells (Figures 2a,b and 5a,b). Early stages of germ cell development are near the testis periphery (Figures 2b and 5b) and advanced stages are located progressively closer to the efferent ducts which contain abundant spermatozoa near the central deferent duct

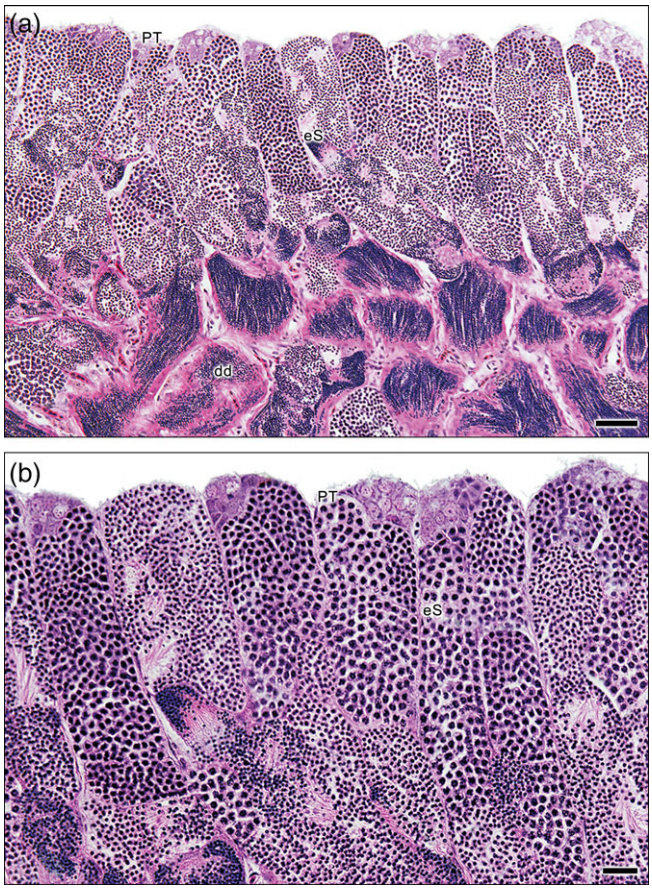

FIGURE 2 *Crenichthys baileyi*, structure of the testis lobules. (a) Longitudinal view of testis lobules and spermatogenesis from the periphery to the center of the testis where the deferent ducts system carry spermatozoa to the exterior. The cysts with germinal cells in early stages of spermatogenesis lie at the periphery of the testis lobules; the later stages of spermatogenesis are progressively closer to the deferent ducts. Bar = 50 μm; (b) Detail of the testis periphery of Figure 2a to demonstrate the germ cells cysts in early stages of spermatogenesis. Bar = 20 μm. dd = Deferent ducts; eS = early stages of spermatogenesis; PT = periphery of the testis; H&E

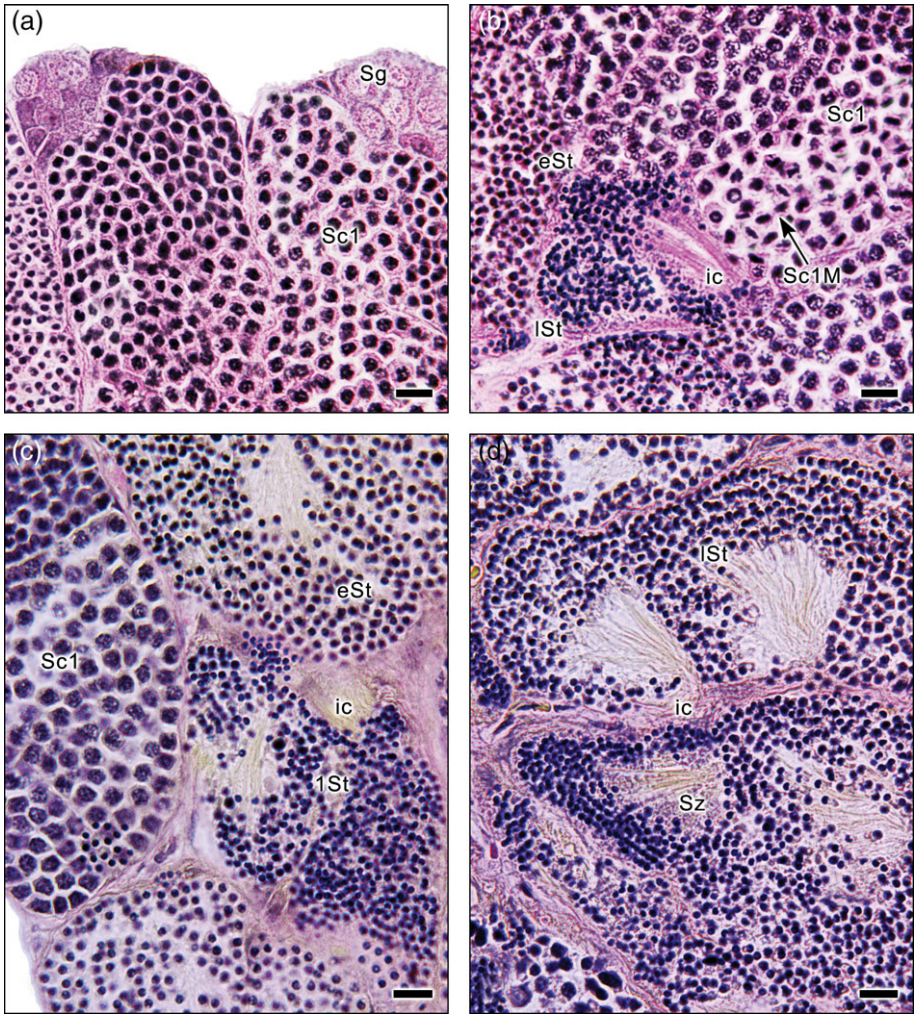

FIGURE 3 *Crenichthys baileyi*, spermatogenesis in the testis to demonstrate germ cells in different stages. The interstitial compartment is observed between the lobules. (a) Groups of spermatogonia are restricted to the periphery of the testis lobules and primary spermatocytes lie proximal to them. (b) Primary spermatocytes during metaphase of the first division of meiosis, with early and late spermatids. Some primary spermatocytes are seen in metaphase (arrow). (c) Groups of early and late spermatids next to primary spermatocytes. (d) Late spermatids and spermatozoa. Bars = 10 μm. eSt = Early spermatids; ic = interstitial compartment; lSt = late spermatids; Sc1 = primary spermatocytes; Sc1M = primary spermatocytes in metaphase; Sg = spermatogonia; Sz = spermatozoa. H&E

(Figures 2a and 5a). Consequently, as germ cells mature during spermatogenesis, the cysts migrate within the lobules toward the efferent duct system and central deferent duct. Within the cyst, the flagella of spermatozoa are arranged irregularly.

3.2 | Spermatogenesis

3.2.1 | Spermatogonia

The spermatogonia are located only at the periphery of the testis (Figures 3a and 6a). They are seen as individual cells or in small groups. The cells are spherical in shape and are the largest of the germ cells, with diameters of 12 to 16 μm. They have a spherical nucleus with one or two nucleoli and the cytoplasm is somewhat granular.

3.2.2 | Primary spermatocytes

Primary spermatocytes are spherical cells, somewhat smaller than the spermatogonia, with diameters of 10 to 12 μm (Figures 3a–c and 6a,b). The nucleus has replicated, homologous chromosomes which are in different stages of Prophase I of meiosis: leptotene (with fine reticular replicated, homologous chromosomes), zygotene (with homologous chromosomes in synapsis), pachytene (with paired homologous chromosomes condensed) and diplotene (with separation of replicated homologous chromosomes that remain attached at the chiasmata). Cysts with primary spermatocytes at the pachytene stage are abundant (Figures 3b,c and 6b,c), whereas those in leptotene, zygotene, and diplotene stage are fewer in number. The primary spermatocytes enter Metaphase I, Anaphase I, and Telophase I, resulting in two secondary spermatocytes.

3.2.3 | Secondary spermatocytes

Secondary spermatocytes have diameters of 5 to 8 μm. They are spherical with a spherical nucleus which contains filamentous chromosomes (Figures 3b and 6c). Because secondary spermatocytes are the result of division of the primary spermatocytes, their

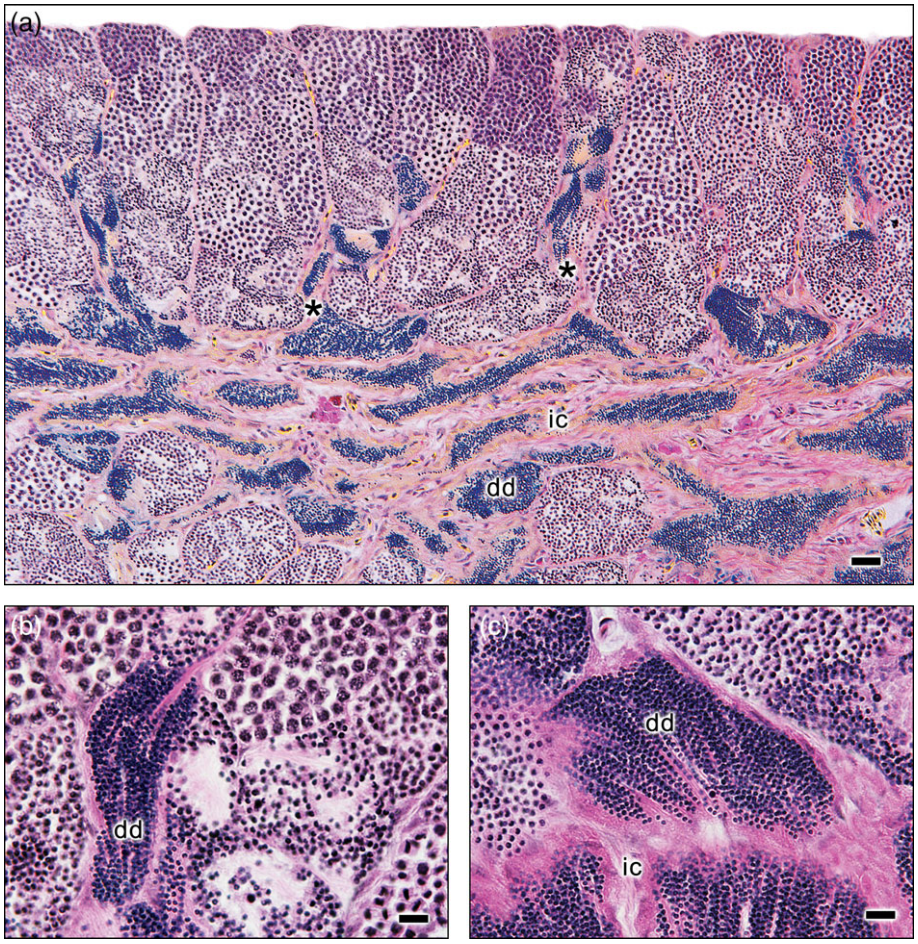

FIGURE 4 *Crenichthys baileyi*, spermiation in the testis. (a) at the proximal end of the lobules, the cysts open and the spermatozoa move into the central deferent duct. Bar = 50 μm. (b) the spermatozoa are numerous, occupying the lumen of the deferent ducts. Bar = 10 μm. (c) the interstitial compartment is prominent between the deferent ducts which are full of spermatozoa. Bar = 10 μm. dd = Deferent ducts; * = proximal end of the lobules; ic = interstitial compartment. H&E

nucleus contains a haploid number of chromosomes, yet duplicated. These spermatocytes are seen rarely in histological preparations because they advance immediately to the second division of meiosis.

3.2.4 | Spermatids

Spermatids have diameters of 3 to 5 μm. Because this cell type is the result of divisions of secondary spermatocytes, spermatids are haploid cells. They are spherical in shape, have a spherical nucleus and undergo morphological changes to become spermatozoa through the process of spermiogenesis (Figures 3a–d and 6a,c,d). Their chromatin becomes condensed, stains more intensely, and the flagellae grow. As spermiogenesis proceeds, the nucleus becomes smaller and denser (Figures 3b–d and 6b,c).

3.2.5 | Spermatozoa

Spermatozoa are the mature cells of spermatogenesis observed at the culmination of spermiogenesis. The head of the spermatozoon is spherical and densely basophilic (Figures 3b,d and 6d). During spermiation, the spermatozoa are released and move into the efferent ducts.

3.2.6 | Spermiation

Spermiation is observed at the proximal end of the lobules when the cysts open and the spermatozoa move to the central deferent duct. The spermatozoa are numerous and fill the lumen of the ducts (Figures 4a–c and 7a–c).

3.3 | Testis of the viviparous goodeid *Ataeniobius toweri*

The testis of viviparous goodeids is like that of *C. baileyi* and *E. latos* in that it is a restricted lobular testis with spermatogonia present only at the distal tips of the lobules (Figure 8a). One marked feature differentiates the testis of the oviparous *C. baileyi* and *E. latos* from the viviparous goodeids represented here by *Ataeniobius toweri*: spermatozoa in the cysts form bundles called spermatozeugmata. They are abundant in the lobules (Figure 8a) near the central deferent duct, and all along the duct (Figures 8b,c).

4 | DISCUSSION

Histological examination of the testes of *C. baileyi* and *E. latos* confirms that they are composed of germinal and interstitial compartments. Both

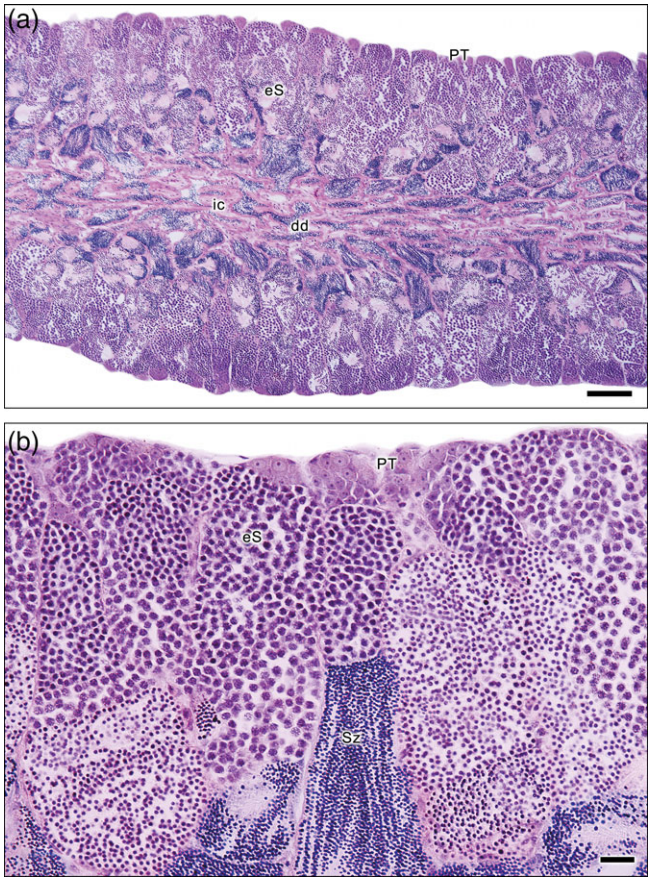

FIGURE 5 *Empetrichthys latos*, structure of the testis lobules. (a) the disposition and development of the spermatogenesis is longitudinal, from the periphery to the center of the testis from where the deferent ducts system carries spermatozoa to the exterior. Cysts with germinal cells in early stages of spermatogenesis are situated at the periphery of the testis lobules. The later stages of spermatogenesis are progressively closer to the deferent ducts. Bar = 100 μm, (b) detail of the periphery of the testis to demonstrate the germ cells cysts in early stages of spermatogenesis. The proximal end of a lobule contains abundant spermatozoa being carried to the deferent duct system. Bar = 20 μm. dd = Deferent ducts;
eS = early stages of spermatogenesis; PT = periphery of the testis; Sz = spermatozoa at the proximal end of a lobule. H&E

compartments are always separated by a basement membrane in all vertebrates (Grier et al., 2016). The germinal compartment is composed of the germinal epithelium formed by two cell types: somatic Sertoli cells and germ cells. Sertoli cells are homologous among all vertebrates, based on their common origin from the germinal ridge (Grier, Uribe, Parenti, & De la Rosa-Cruz, 2005; Grier and Uribe, 2009).

In *C. baileyi* and *E. latos*, as in other goodeids (Mazzoldi, Lorenzi, & Rasotto, 2007; Nelson, 1975), testes are paired, elongate organs that are attached to the dorsal wall of the body by a mesorchium. Both testes join caudally to converge in one central deferent duct. Where the deferent ducts emerge from the testes, they immediately join and fuse into a common sperm duct, establishing the vas deferens which is open to the exterior through the urogenital pore.

The interstitial compartments observed in the testes of *C. baileyi* and *E. latos* are composed of connective tissue (fibroblasts, immunological cells as macrophages and lymphocytes, collagen fibers, myoid cells, blood vessels, and nerve fibers) and Leydig cells, as in all teleosts (Grier, 1993).

The arrangement of the testicular germinal epithelium varies among bony fishes. "Basal" osteichthyans, such as coelacanths, paddlefishes, gars, and ancestral teleosts have a testis formed of anastomosing tubules that do not terminate at the testis periphery, but, instead, form interconnected loops (Parenti & Grier, 2004: their Table 1). More derived taxa have a lobular testis, proposed as a morphological synapomorphy of neoteleost fishes (Parenti & Grier, 2004). The lobular testis occurs in two types based on the distribution and arrangement of spermatogonia. In nonatherinomorph neoteleosts, the testis is an unrestricted spermatogonial lobular testis in which spermatogonia and spermatocysts, at various stages of development, are distributed along the length of the lobules. In contrast, atherinomorph fishes possess a restricted spermatogonial lobular testis type in which lobules show a progression of developing germ cells, with spermatogonia confined to the testicular periphery (Jamieson, 1989; Grier, 1993; Parenti & Grier, 2004; Parenti, 2005; Grier and Uribe, 2009; Uribe et al., 2015). This restricted spermatogonial lobular testis type is well defined in *C. baileyi* and *E. latos* as in all atherinomorphs, including all goodeins. The identification of a restricted lobular testis in the empetrichthyins is additional support of atherinomorph monophyly (Parenti, 2005; Parenti & Grier, 2004).

Spermatogenesis of *C. baileyi* and *E. latos* is similar to that described in bony fishes and other vertebrates (Grier, 1981, 1993; Uribe et al., 2015). This complex process is initiated by the mitotic proliferation of spermatogonia, proceeds through meiosis in two divisions, and concludes with spermiogenesis during which the haploid spermatids transform morphologically into motile, flagellated spermatozoa.

Spermatogenesis is fundamentally different in teleosts and other anamniotes compared to amniotes, as seen in *C. baileyi* and *E. latos*. Teleosts have a cystic condition or develop a spermatocyst (Billard, 1986; Grier, 1993), defined by Sertoli cell processes surrounding an isogenic clone of germ cells. During spermatogenesis, the number of developing sperm within a spermatocyst increases greatly due to several mitotic divisions of the spermatogonia followed by meiosis of the spermatocytes. The cystic condition permits Sertoli cells to provide nutrients and a permeability barrier throughout spermatogenesis (Billard, 1986; Grier, 1993). The cystic arrangement of germ cells seen in *C. baileyi* and *E. latos* is like that of all teleosts.

We describe one major difference in reproductive morphology between males of the two subfamilies of goodeids. The viviparous goodeids discharge semen in large aggregates of cysts filled with spermatozoa, called spermatozeugmata, into the deferent ducts (spermiation) whereas empetrichthyins release "free sperm." The presence of spermatozeugmata in goodeins has been documented previously (Grier et al., 1978; Uribe et al., 2009, 2015). In species of the subfamily Goodeinae, as in *Ilyodon whitei* (see Grier et al., 1978; Grier and Uribe, 2009), *Xenotoca eiseni* (see Grier and Uribe, 2009; Liu, Torres, & Tiersch, 2018a, 2018b; Uribe et al., 2015), *Goodea atripinnis* (see Liu et al., 2018a; Uribe et al., 2015) and *Ataeniobius toweri* (see Liu et al., 2018a), large groups of cysts with spermatozoa are bundled in spermatozeugmata in the testis and in the deferent ducts. The

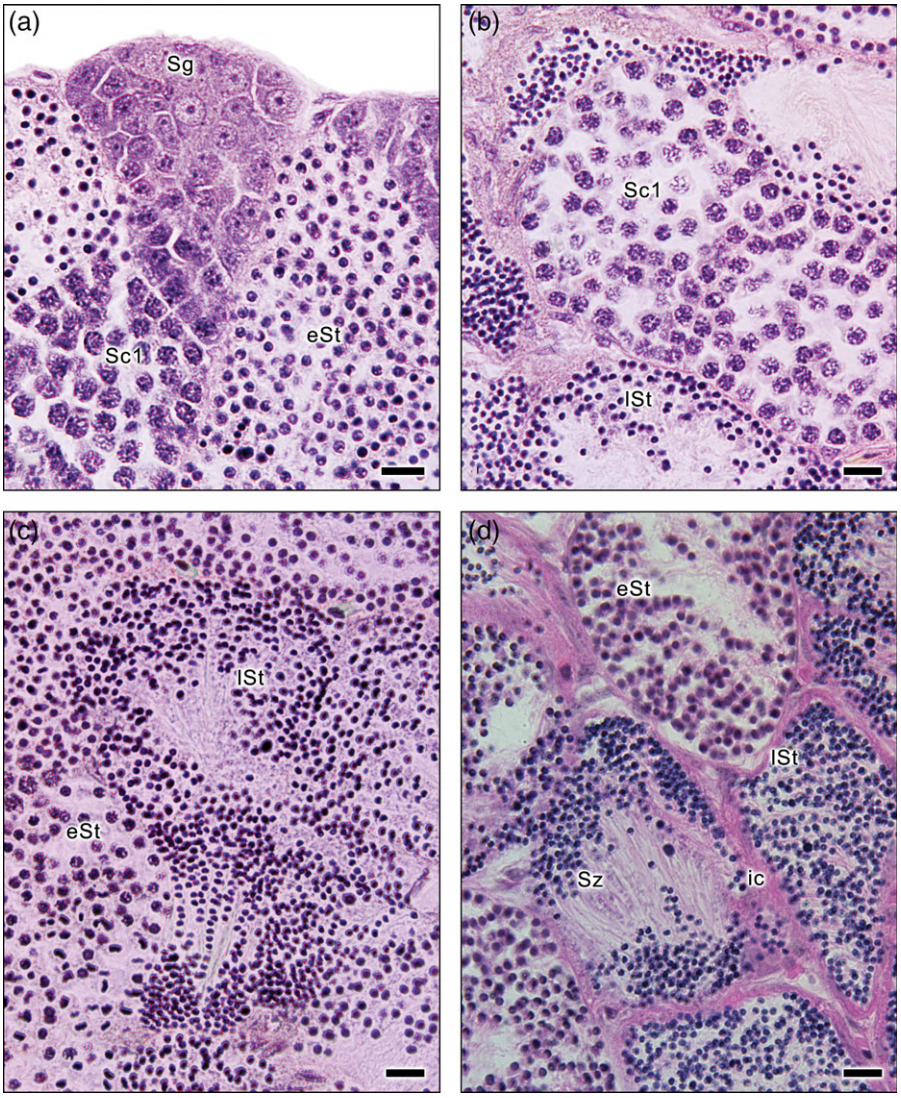

FIGURE 6 *Empetrichthys latos*, spermatogenesis in the testis. (a) Groups of spermatogonia at the periphery of the testis. Cyst with germ cells in different stages of spermatogenesis: Primary spermatocytes, early spermatids. (b) Primary spermatocytes and late spermatids. (c) Early and late spermatocytes. (d) Early and late spermatocytes and spermatozoa. The interstitial compartment is observed between the lobules. Bars = 10 μm. eSt = Early spermatids (eSt); ic = interstitial compartment; lSt = late spermatids; Sc1 = primary spermatocytes; Sg = spermatogonia; Sz = spermatozoa. H&E

spermatozeugmata are formed by an enveloping layer of mucotic substance secreted by the Sertoli cells (Fishelson, Gon, Holdengreber, & Delarea, 2007). In viviparous species, spermatozeugmata are passed from males to females; fertilization is internal and females give birth to live young. Spermatozeugmata provide efficient transfer of spermatozoa to the female reproductive system to accomplish internal fertilization, minimizing the dramatic loss of sperm that occurs when sperm are broadcast into the environment.

The mechanism of internal fertilization in viviparous goodeids is not well understood. In those taxa for which sperm morphology has been reported, viviparous goodeids have an anacrosomal aquasperm typical of oviparous cyprinodontiforms (Grier et al., 2005; Jamieson, 2009). In contrast, viviparous poeciliids have a sperm with an elongated nucleus and midpiece, typical of viviparous taxa (Jamieson, 2009). Further, viviparous goodeid males do not have a gonopodium as do the viviparous poeciliids; the anal fin is divided into an anterior and a posterior portion without elongation and elaborate modification of anal-fin rays. During mating, as the male and female pair, the male viviparous goodeid wraps the anterior portion of his anal fin around the female near the gonopore to form a conduit through which spermatozeugmata are passed (Nelson, 1975). In the anal fin of all adult male goodeids, both oviparous and viviparous, the first two to seven middle anal radials are fused to the proximal radials, a diagnostic character that separates them from all other killifishes (Parenti, 1981: her Figure 71). Coupling during mating is common among killifishes; how the modified anal fin of male empetrichthyins might facilitate fertilization is unknown.

One generalization for teleosts is that viviparous species form spermatozeugmata and that oviparous species release “free sperm” (Grier et al., 1978; Jamieson, 1989; Liu et al., 2018a). Yet this is not

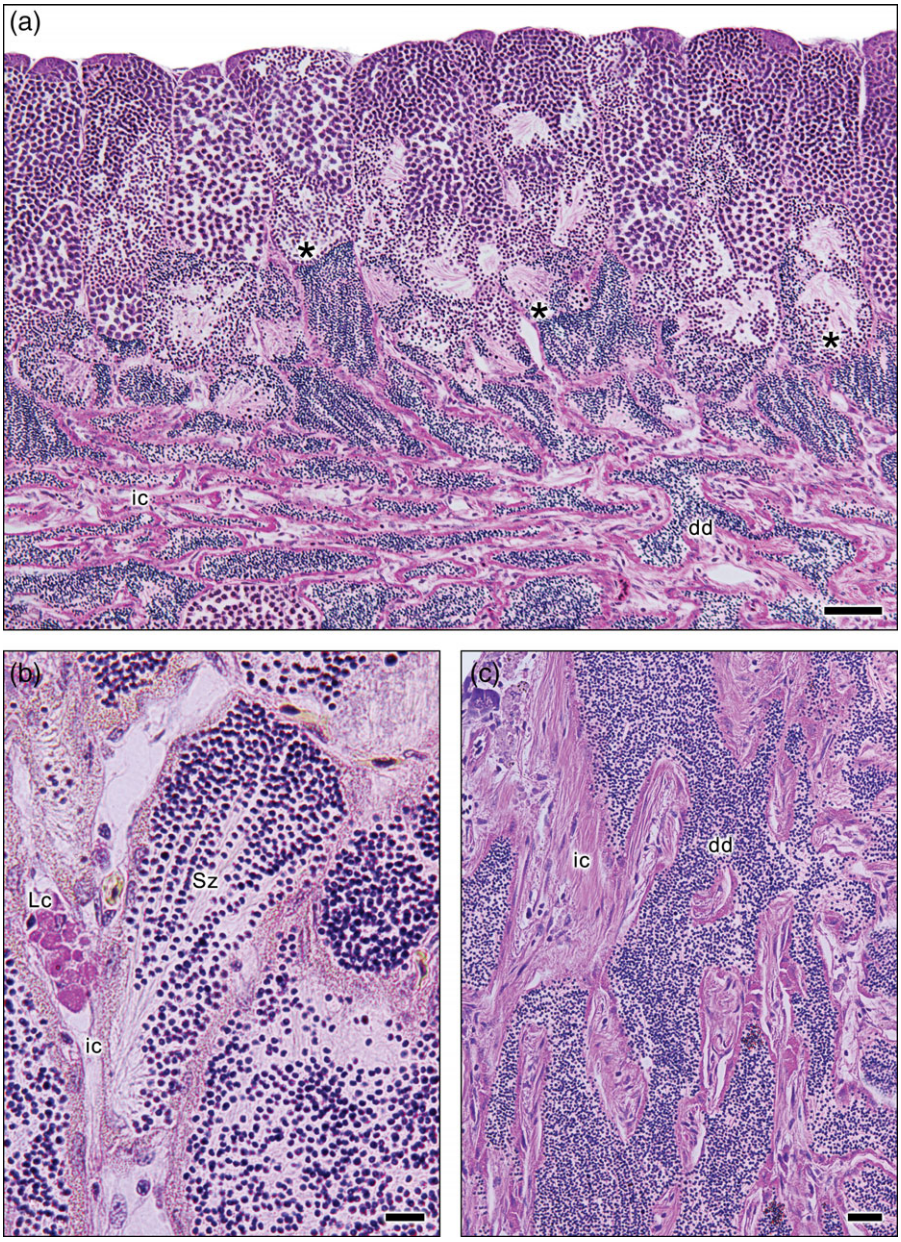

FIGURE 7 *Empetrichthys latos*, spermiation in the testis. (a) at the proximal end of the lobules, the cysts open and the spermatozoa move into the central deferent duct. Bar = 50 μm. (b) the spermatozoa are numerous, occupying the lumen of the ducts. A group of Leydig cells are seen (Lc). B = 10 μm. (c) the interstitial compartment is prominent between the deferent ducts. Bar = 20 μm. dd = Deferent ducts; * = proximal end of the lobules; ic = interstitial compartment; Lc = Leydig cells. H&E

true in all cases (e.g., Mann, 1984). For example, most viviparous cyprinodontiforms in the genera *Anableps* and *Jenynsia* do not form sperm bundles, but release free sperm into a fleshy, tubular, gonopodium through which the sperm are transferred to the female; fertilization is internal and females give birth to live young (Grier, Burns, & Flores, 1981). In the atheriniform phallostethids, males form spermatozeugmata which are transferred to the females through an elaborate intromittent organ; fertilization is internal and females lay fertilized eggs (Grier & Parenti, 1994; Parenti, 1989). Therefore, it was necessary to confirm how male empetrichthyins release sperm: in both *C. baileyi* and *E. latos*, spermatozeugmata are not formed; they have "free sperm" as in many other oviparous teleost species.

Populations of both species *C. baileyi* and *E. latos* are extinct, endangered or threatened in nature (Gilbert, 1893; Grant & Riddle, 1995; Minckley and Deacon, 1968; Minckley and Marsch, 2009). As with our study of the ovarian structure and oogenesis of females of both species (Uribe et al., 2012), access to well-fixed archival museum specimens has allowed us to study essential aspects of their reproduction. Our documentation of the testis morphology and spermatogenesis in two oviparous species of goodeids, *C. baileyi* and *E. latos*, adds to our understanding of the biology of these remarkable and important species, and to our growing body of knowledge about the evolution of reproductive biology in oviparous and viviparous atherinomorph fishes.

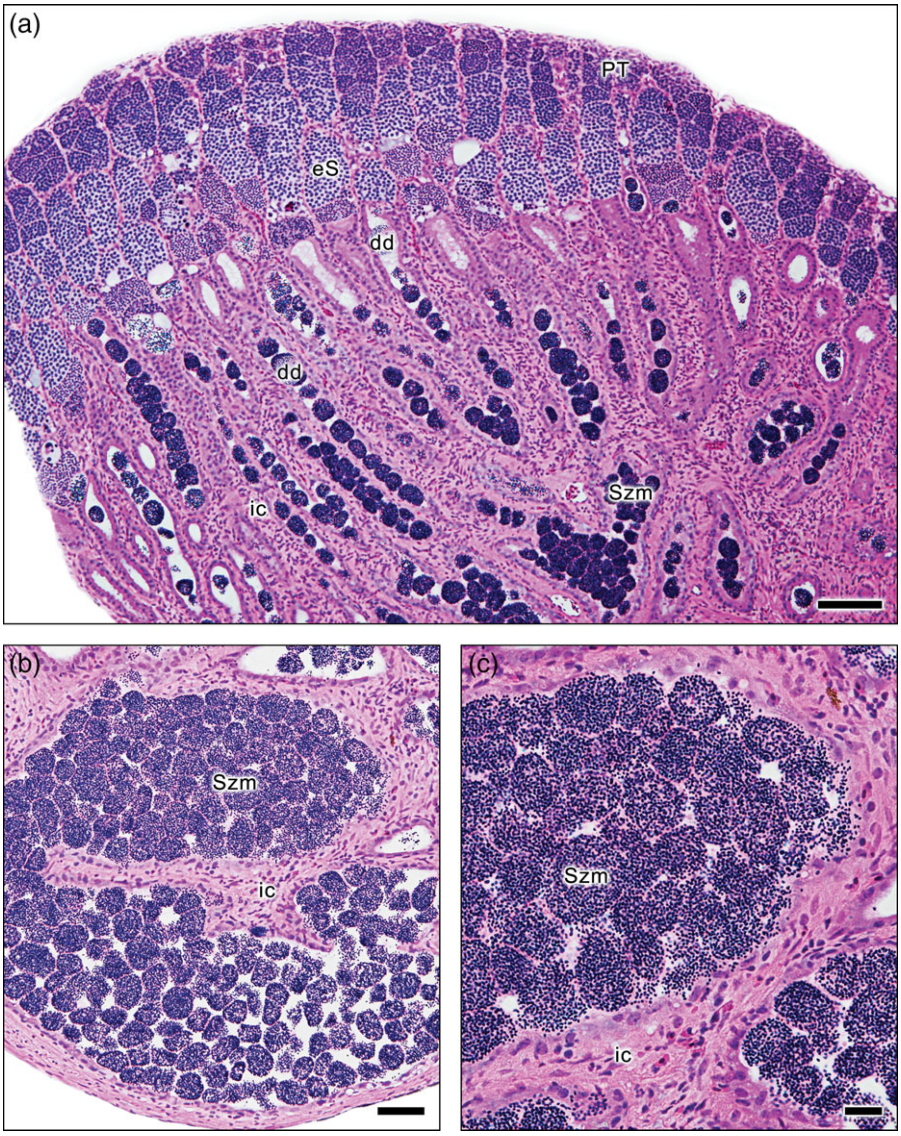

FIGURE 8 *Ataeniobius toweri*, testis lobes of viviparous goodeid. (a) the development of the spermatogenesis proceeds from early stages of spermatogenesis near the periphery to spermatozeugmata at the center of the testis. The cystic form of the germ cells is evident. Bar = 100 μm. (b) the spermatozoa are bundled in spermatozeugmata within the cysts in the testis lobules and in the deferent ducts. Bar = 50 μm. (c) a close-up of the middle of 8B to demonstrate the abundant spermatozoa in the bundles (spermatozeugmata) in interstitial compartments. Bar = 20 μm. dd = Deferent ducts; eS = early stages of spermatogenesis; ic = interstitial compartment; PT = periphery of the testis; Szm = spermatozeugmata. H&E plus PAS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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