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Reproductive Histology of *Tomeurus gracilis* Eigenmann, 1909 (Teleostei: Atherinomorpha: Poeciliidae) With Comments on Evolution of Viviparity in Atherinomorph Fishes

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ABSTRACT *Tomeurus gracilis* is a species long considered pivotal in understanding the evolution of livebearing in atherinomorph fishes. *Tomeurus gracilis* is a zygoparous or embryoparous poeciliid: internal fertilization is followed by females laying fertilized eggs singly or retaining fertilized eggs until or near hatching. *Tomeurus* was hypothesized as the sister group of the viviparous poeciliids until it was proposed as a close relative of a derived viviparous poeciliid, *Cnesterodon*, hence nested among viviparous taxa rather than near the root of the tree. Here, we describe and compare reproductive morphological characters of the little-known *Tomeurus* with those of representative atherinomorphs. In *Tomeurus* and *Cnesterodon*, sperm are packaged in naked sperm bundles, or spermatozeugmata, in a configuration considered here diagnostic of viviparous poeciliids. Testes are single and free sperm are stored in the ovary in both taxa in contrast to oviparous atherinomorphs in which testes are paired and sperm are not packaged and not stored in the ovary. Efferent ducts in *Cnesterodon* testes and other viviparous poeciliids have a PAS-positive secretion demonstrating presence of a glycoprotein that inactivates sperm or prevents final sperm maturation. No PAS-positive staining secretion was observed in *Tomeurus* or oviparous atherinomorphs. *Tomeurus* shares apomorphic reproductive characters, such as sperm bundle and testis morphology and a gonopodium, with viviparous poeciliids and plesiomorphic characters, such as a thick zona pellucida with filaments, with oviparous taxa. We do not postulate loss or reversal of viviparity in *Tomeurus*, and we corroborate its phylogenetic position as sister to the viviparous poeciliids. J. Morphol. 271:1399–1406, 2010.

KEY WORDS: spermatozeugma; zona pellucida; egg morphology; embryoparity/zygoparity; testis types

INTRODUCTION

Atherinomorph fishes, with an estimated 1,552 species classified in three orders, Atheriniformes, Cyprinodontiformes, and Beloniformes, have long been featured in studies of reproductive biology (see Parenti, 2005; Nelson, 2006). Atherinomorph monophyly is well-supported by a range of morphological characters (Rosen, 1964; Rosen and Parenti, 1981; Parenti, 1993, 2005) and has been recovered in molecular analyses of bony fish phylogeny (e.g., Setiamarga et al., 2008). Two diagnostic characters of atherinomorphs are explicitly of reproductive morphology: a testis with spermatogonia restricted to the distal ends of testis lobules rather than distributed along the length of the lobule and a relatively large egg with fluid rather than granular yolk (Grier et al., 1980; Rosen and Parenti, 1981; Grier, 1993; Parenti and Grier, 2004). Internal fertilization and viviparity have evolved multiple times within atherinomorphs, as interpreted from the most parsimonious distribution of morphological and molecular characters, many of which are not related directly to reproduction (see Rosen, 1964; Parenti, 1981, 1993, 2005; Rosen and Parenti, 1981; Meyer and Lydeard, 1993; Grier et al., 2005; Mank and Avise, 2006; Hrbek et al., 2007; Reznick et al., 2007a).

Tomeurus gracilis Eigenmann (1909) (Fig. 1A) is a diminutive, zygoparous or embryoparous atherinomorph poeciliid fish that lives in northeastern South America. A simple, straightforward classifi-

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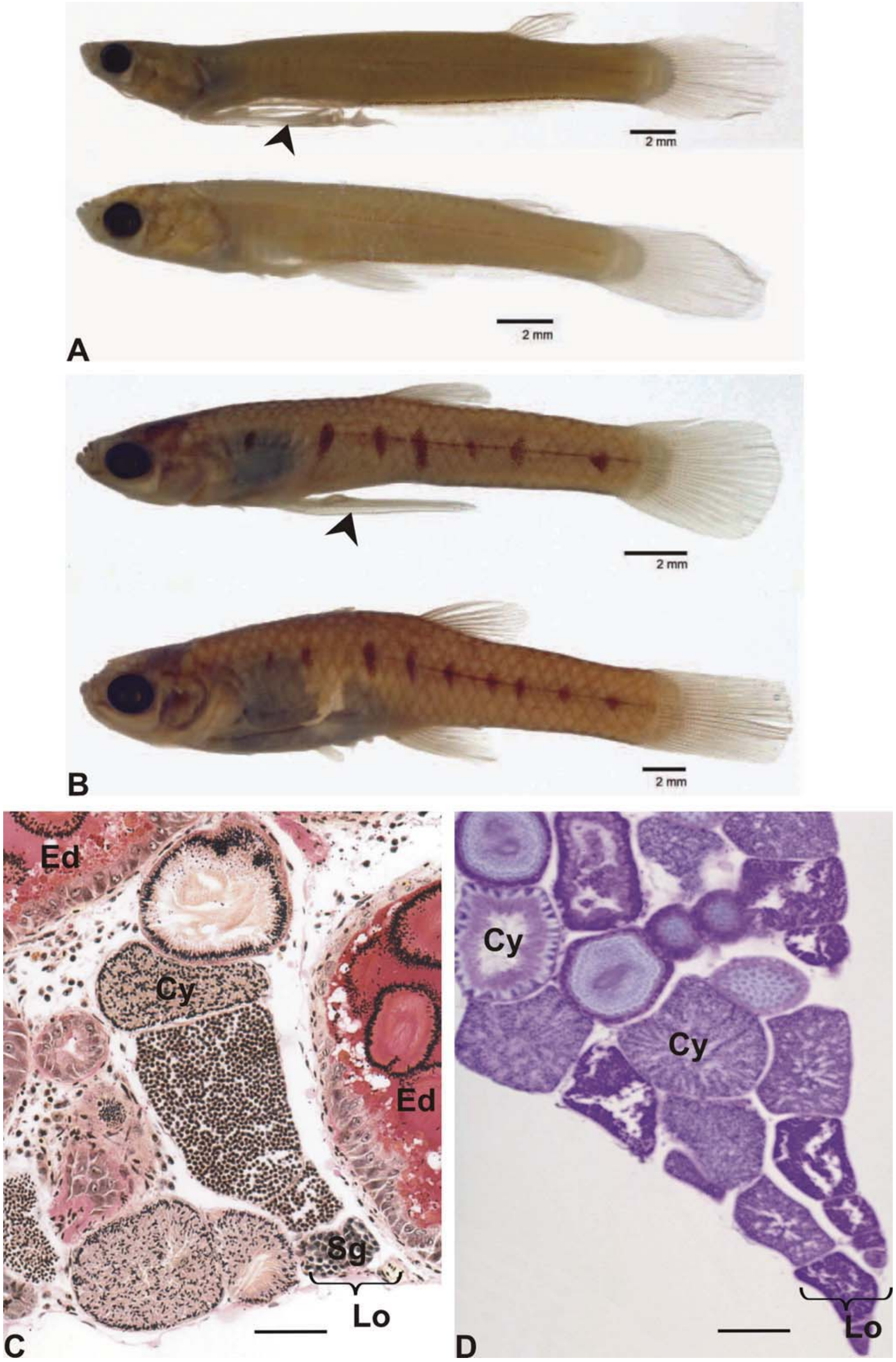

Fig. 1. **A**: *Tomeurus gracilis*, USNM 225464, Surinam. Male, above; female, below. Arrowhead points to gonopodium. Scale bar = 2 mm. **B**: *Cnesterodon decemmaculatus*, USNM 360480, Argentina. Male, above; female, below. Arrowhead points to gonopodium. Scale bar = 2 mm. **C** and **D**: Transverse sections through testes of mature males. C: *Cnesterodon decemmaculatus*, USNM 360480, 21.5 mm SL. D: *Tomeurus gracilis*, USNM 225463, 25 mm SL. In both testes, primary spermatogonia (Sg) are restricted to the distal termini of the lobules (Lo; brackets), just beneath the tunica albuginea. These produce secondary spermatogonia, organized into cysts (Cy) that progress toward the center of the testis, location of the efferent ducts (Ed), during spermatogenesis. C, periodic acid/Schiff-metanil yellow-hematoxylin, D, thinonin. Scale bar = 50 µm.

cation of reproductive mode was advocated by Wourms (1981): oviparous (lays eggs) versus viviparous (gives birth to live young). We follow other fish reproductive biologists and recognize three levels of oviparity: ovuloparity, ova are fertilized and develop externally; zygoparity, ova are fertilized internally and held for a relatively short time before being released; and embryoparity, ova are fertilized internally and embryos may become well developed before being released (see Muñoz et al., 2002:877). Zygoparity or embryoparity are more precise than ovoviviparity, a term used to describe a state between oviparity and viviparity.

When first described, *Tomeurus* was identified as "... the type of a new subfamily of poeciliids" (Eigenmann, 1909:53), at once recognized as related to the known poeciliids, yet strikingly different from them. Observations on the reproductive biology of *Tomeurus*, many largely anecdotal, were summarized by Breder and Rosen (1966). Despite the potential importance of *Tomeurus* in understanding the evolution of viviparity in atherinomorph fishes, little is known about the basic details of its reproductive biology, such as embryonic development, reproductive behavior, or physiology (Schories and Schartl, 2005). Its rarity in museum collections and the aquarium trade has meant its omission from some phylogenetic studies of poeciliid fishes (viz. Bisazza et al., 1997).

Tomeurus males transfer sperm bundles to the female via a gonopodium; sperm bundles have been seen attached to the female near the gonopore (Gordon, 1955). Subsequent internal fertilization is followed by females laying fertilized eggs (Gordon, 1955; Rosen and Bailey, 1963). In part because *Tomeurus* females lay fertilized eggs and are not livebearers, or facultatively retain embryos until or close to hatching, this monotypic genus had long been hypothesized as the closest living relative of the viviparous poeciliids (e.g., Rosen and Bailey, 1963).

Using comparative morphology, Ghedotti (2000) proposed that *Tomeurus* is a close relative of the viviparous poeciliid genus *Cnesterodon* (Fig. 1B) of southeastern South America (Lucinda, 2005). *Tomeurus* was nested among viviparous poeciliids as a member of the tribe Cnesterodontini in this phylogenetic hypothesis of poecilioid relationships (Ghedotti 2000: fig. 21c). The *Tomeurus-Cnesterodon* sister group relationship was implied also in several preliminary molecular phylogenetic analyses by Parker (1997). In contrast, the traditional placement of *Tomeurus* as sister group of the viviparous poeciliids was corroborated in a molecular phylogenetic analysis by Meyer and Lydeard (1993), and in a morphological phylogenetic analysis by Lucinda and Reis (2005) who explicitly reinterpreted homology of some of the characters described by Ghedotti (2000). A recent molecular perspective on viviparous poeciliid phylogeny presented a third alternative, that the highly autapomorphic viviparous *Xenodexia* Hubbs (1950) is sister to a clade comprising *Tomeurus* as sister to the remaining viviparous poeciliids (Hrbek et al., 2007).

Here, we describe a range of reproductive characters, principally those demonstrated via histology, to ask if *Tomeurus* has characters of oviparous or viviparous taxa and, further, whether these are homologs or homoplasies. Our goal is to better understand the evolution of reproductive modes within atherinomorphs and the phylogenetic position of *Tomeurus*.

MATERIALS AND METHODS

Tomeurus gracilis specimens were collected in Surinam in 1980, preserved in 10% formalin and transferred to 75% ethanol for long-term storage. *Cnesterodon decemmaculatus* (Jenyns, 1840–1842) specimens were collected in Buenos Aires, Argentina in 1999; gonads were fixed in 2% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer, pH 7.3, and voucher specimens preserved in formalin and transferred to 75% ethanol. Specimens reported on here (referred to by USNM catalog numbers) and additional voucher materials, including histological slides, are housed in the Division of Fishes, National Museum of Natural History, Smithsonian Institution. Gonads were embedded in plastic (JB-4 embedding kit, Polysciences, USA), sectioned at 4 μm and stained with periodic acid/Schiff-metanil yellow-hematoxylin (Quintero-Hunter et al., 1991), toluidine blue, or thionin. Micrographs were taken with a Nikon-Microphot FX microscope.

RESULTS

Testis and Sperm

Vertebrate testes are ancestrally paired, symmetrical organs that lie along the dorsal body wall on either side of the gut. *Tomeurus gracilis* has a single testis, with no morphological evidence of fusion of paired testes. In *Cnesterodon decemmaculatus*, the testes are fused along the midline into a single organ, as reported for other viviparous poeciliids (van den Hurk, 1973).

Spermatogonia are restricted to the termini of the testis lobules in *Tomeurus gracilis* and *Cnesterodon decemmaculatus* (Fig. 1C,D), as in all atherinomorphs, as far as known (Grier et al., 1980; Parenti and Grier, 2004). "Restricted" testes demonstrate a maturational progression of germ cell stages toward the efferent ducts. Primary spermatogonia are restricted to the distal termini of the lobules just beneath the tunica albuginea. Primary spermatogonia produce secondary spermatogonia organized into cysts that move toward the center of the testis during spermatogenesis. Upon completion of meiosis, sperm nuclei elongate and become embedded in Sertoli cell cytoplasmic recesses. At spermiation, each spermatocyst releases a single spermatozeugma, or sperm bundle (Fig. 2A,B,D,E). These unencapsulated or naked sperm bundles are distinguished by elongate sperm nuclei oriented toward the periphery and flagella toward the center (Fig. 2A,B,D,E). In

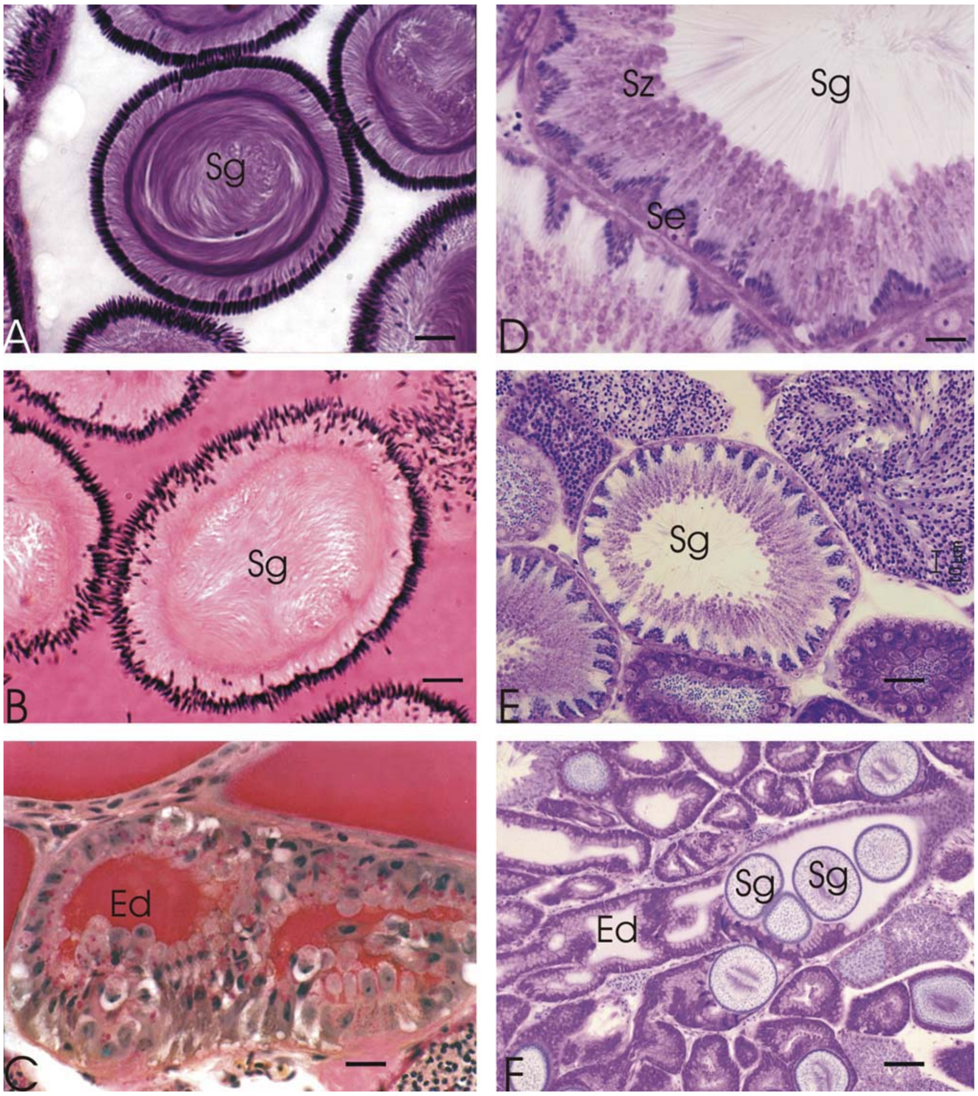

Fig. 2. Sections through testes of mature male *Cnesterodon decemmaculatus* (**A**, **B**, **C**) and *Tomeurus gracilis* (**D**, **E**, **F**). Upon completion of meiosis, sperm (Sz) nuclei elongate and become embedded in Sertoli cell cytoplasmic processes (Se). At spermiation, each spermatocyst releases a single spermatozeugma (Sg). These naked sperm bundles have elongated sperm nuclei oriented toward the periphery and flagella toward the center. In *Tomeurus*, the nuclei are arranged in triangular groups (D, E). In *Cnesterodon* (B, C), spermatozeugmata (Sg) are immersed in a pink PAS-positive substance secreted by the efferent ducts (Ed) indicating presence of glycoproteins that keep sperm inactive or prevent final sperm maturation. In *Tomeurus* (E, F), PAS-negative substance is secreted by the efferent ducts. A, toluidine blue; B, C, E and F, periodic acid/Schiff-metanil yellow-hematoxylin. D, thionin. Scale bar = 10 μm.

Tomeurus, the nuclei are arranged in distinctive, triangular groups (Fig. 2D,E).

Spermatozeugmata in efferent ducts of *Cnesterodon* are immersed in a PAS-positive, pink glycoprotein (Fig. 2B,C), demonstrating the presence of a secretion by duct cells. *Tomeurus* efferent ducts are PAS-negative; there is no uptake of pink stain (Fig. 2E,F).

Ovary and Ovum

The ovary of both *Tomeurus gracilis* and *Cnesterodon decemmaculatus* is a single, median organ. They are cystovarian: ovulation is into the ovarian lumen. Fertilization is internal and free sperm are stored in the ovary (Fig. 3A,B, insets).

The zona pellucida (chorion or vitelline envelope) of *Tomeurus* is relatively thick and has adhesive filaments on the outer surface with which the fertilized ovum may be attached to vegetation (Fig. 3A; see Rosen and Bailey, 1963:fig. 8). In contrast, the zona pellucida of *Cnesterodon* is so thin as to be barely identifiable and there are no adhesive filaments on the ovum (Fig. 3B, inset). Rosen and Bailey (1963) accurately described the egg of *Tomeurus* with a thick zona pellucida or chorion and adhesive filaments; they erroneously described the zona pellucida as absent in viviparous poeciliines.

Mature oocytes of both *Tomeurus* and *Cnesterodon* have fluid, rather than granular yolk, a diag-

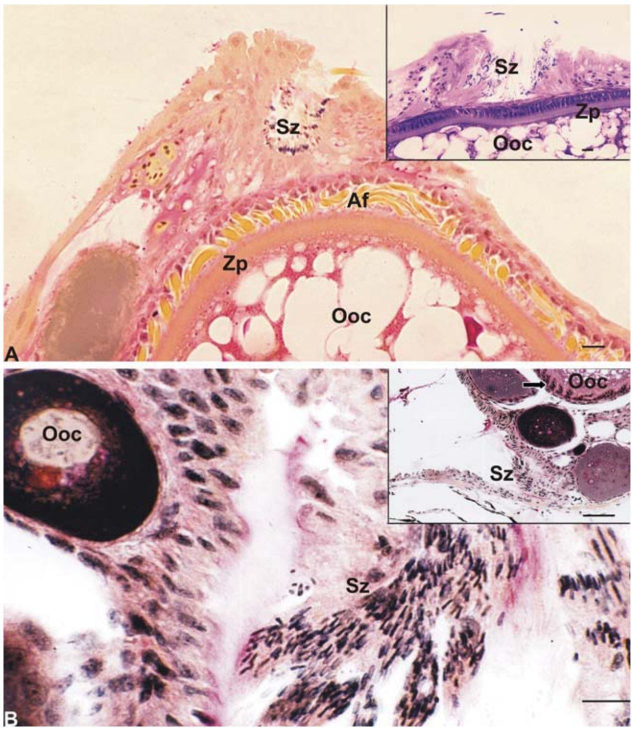

Fig. 3. Sections through ovaries of mature females. **A**: *Tomeurus gracilis*, USNM 225463, 20 mm SL. **B:** *Cnesterodon decemmaculatus*, USNM 360480, 26.5 mm SL. Fertilization of oocytes (Ooc) is internal and free sperm (Sz) are stored in the ovary in both species [figures and insets]. The zona pellucida (Zp) of *Tomeurus* is relatively thick and there are many adhesive filaments (Af) on the surface, whereas that of *Cnesterodon* (black arrow in inset) is extremely thin and lacks adhesive filaments. All figures periodic acid/Schiff-metanil yellow-hematoxylin. Scale bar = 10 μm.

nostic character of atherinomorphs (Parenti and Grier, 2004).

DISCUSSION

One, unambiguous reproductive histological homology or synapomorphy supports the close relationship of *Tomeurus gracilis* to the viviparous poeciliids: sperm bundle morphology (Table 1). In both *Tomeurus* and *Cnesterodon,* sperm are packaged in naked sperm bundles, or spermatozeugmata, that are morphologically similar to those of the viviparous poeciliids as far as known: sperm nuclei are oriented toward the periphery and flagella toward the center of the bundle. This is proposed here as a diagnostic character or synapomorphy of the subfamily Poeciliinae *sensu* Parenti (1981).

Sperm bundles are formed in other internally fertilizing atherinomorphs; these are not homologs at the hierarchical level of the Poeciliinae. Viviparous members of the atherinomorph cyprinodontiform family Goodeidae, for example, have spermatozeugmata with flagella oriented toward the pe-

TABLE 1. Comparison of select characters among five atherinomorph taxa exhibiting various reproductive modes

Tomeurus[a]	*Cnesterodon*[b]	*Oryzias setnai*[c]	Phallostethinae[d]	*Fundulus*[e]
Cyprinodontiformes	Cyprinodontiformes	Beloniformes	Atheriniformes	Cyprinodontiformes
NE South America; brackish water	SE South America; fresh and brackish water	W India; brackish water	SE Asia; fresh and brackish water	N and M America, Bermuda, Cuba; fresh and brackish water
Modified anal fin in males	Modified anal fin in males	Modified anal fin in males	Modified pectoral and pelvic fin in males	Unmodified fins in males
Internal fertilization	Internal fertilization	Internal fertilization	Internal fertilization	External fertilization
Females lay fertilized eggs; zygoparous or embryoparous	Livebearing; viviparous	Females lay fertilized eggs; zygoparous	Females lay fertilized eggs; zygoparous	Females lay unfertilized eggs; oviparous
Single testis	Single testis	Single, bulb-shaped testis	Single testis	Paired testis
Spermatozeugmata, with sperm nuclei toward periphery of bundle	Spermatozeugmata, with sperm nuclei toward periphery of bundle	Spermatophores (encapsulated sperm bundles)	Spermatozeugmata, with sperm nuclei toward one side of bundle	Free sperm; no sperm bundles formed
Efferent ducts with PAS-negative secretion	Efferent ducts with PAS-positive secretion	Efferent ducts with PAS-negative secretion	Efferent ducts with PAS-negative secretion	Efferent ducts with PAS-negative secretion
Free sperm in ovary	Free sperm in ovary	Free sperm in ovary	Free sperm in ovary	No free sperm in ovary
Ova with thick zona pellucida and adhesive filaments	Ova with thin zona pellucida and no adhesive filaments	Ova with thick zona pellucida and adhesive filaments	Ova with thick zona pellucida and adhesive filaments	Ova with thick zona pellucida and adhesive filaments

[a] This study, Gordon (1955), Rosen and Bailey (1963), and Nelson (2006).
[b] This study, Rosen and Bailey (1963), and Lucinda (2005).
[c] Kulkarni (1940), Grier (1984), Parenti and Grier (2004), Nelson (2006), and Parenti (2008).
[d] Grier et al. (1980) and Grier and Parenti (1994).
[e] Grier et al. (1980) and Parenti and Grier (2004).

riphery and sperm nuclei toward the center (Grier et al., 1978). The zygoparous atherinomorph phallostethids form sperm bundles of yet another, distinct morphology: sperm nuclei are oriented toward one side of the bundle rather than along the periphery (Grier and Parenti, 1994; Table 1). Viviparous halfbeaks of the genera *Dermogenys*, *Nomorhamphus*, and *Hemirhamphodon* form sperm bundles in which sperm nuclei are oriented toward one side of the bundle, with flagella clumped together on the opposite side, suggesting a flower bouquet (Downing and Burns, 1995). *Oryzias setnai* (Kulkarni, 1940), the highly autapomorphic adrianichthyid from western India, is the first atherinomorph documented to have true spermatophores, or encapsulated sperm bundles (Kulkarni, 1940; Grier, 1984; Parenti, 2008). Spermatophores were reported subsequently in several species of the internally fertilizing halfbeak *Zenarchopterus*, yet their development differs from those of *O. setnai*: spermatophores of *O. setnai* are formed in testicular lobule cysts, whereas in *Zenarchopterus* they are formed within the aspermatogenic posterior part of the testis (Grier and Collette, 1987:310–311). Poeciliid sperm bundles were incorrectly called spermatophores by Gordon (1955) and Breder and Rosen (1966), among others.

Cnesterodon testes are fused along the midline to form a single structure that lies against the dorsal body wall, dorsal to the gut, as in all viviparous poeciliids as far as known (van den Hurk, 1973; Grier, 1984). *Tomeurus* has a single testis as do the zygoparous phallostethines (Grier and Parenti, 1994; Table 1). We see no morphological evidence of ontogenetic fusion of paired testes to form a single testis in *Tomeurus*. Likewise, *Oryzias setnai* has a single, bulb-shaped testis, with no evidence of fusion of paired testes (Kulkarni, 1940; Grier, 1984). Homology of these single, median testes within atherinomorphs needs to be tested with, for example, developmental data to see if they form in the same way. These single testes contrast with those of other atherinomorphs such as the viviparous goodeids and anablepids, oviparous fundulids, and oviparous and viviparous zenarchopterids (Aschliman et al., 2005) in which paired testes lie on either side of the gut (see Grier, 1984; Downing and Burns, 1995; Table 1).

Tomeurus and *Cnesterodon* both have a single, median ovary, as do viviparous and oviparous atherinomorphs, as opposed to paired, bilaterally symmetric ovaries (e.g., as in the percomorph *Scorpaena notata*; Muñoz et al., 2002). Free sperm are stored in the ovary in *Tomeurus*, *Cnesterodon* and other internally fertilizing atherinomorphs. In contrast, in oviparous atherinomorphs, such as *Fundulus*, sperm are not packaged and not stored in the ovary (Table 1). All atherinomorphs are cystovarian: they ovulate into the ovarian lumen.

Efferent ducts in *Cnesterodon* and other viviparous poeciliids have a PAS-positive secretion identifying a glycoprotein that keeps sperm inactive and preserves integrity of the sperm bundle or prevents final sperm maturation (van den Hurk and Barends, 1974; Grier, 1981; Fig. 2B,C). The PAS-positive secretion has also been reported in some internally fertilizing halfbeaks of the genera *Zenarchopterus* (Grier and Collette, 1987), and *Dermogenys*, *Hemirhamphodon*, and *Nomorhamphus* (Downing and Burns, 1995). The efferent duct secretion is not PAS-positive in *Tomeurus* (Fig. 2E,F) or any oviparous atherinomorph, as far as known. Keeping the sperm bundle intact or preventing final sperm maturation may be correlated with superfetation: fertilization of successive broods of eggs that are held in successive stages of development (e.g., Reznick and Miles, 1989).

Further, eggs of oviparous atherinomorphs have a relatively thick zona pellucida and adhesive filaments by which fertilized eggs are attached to plants or embryos are clumped together during development. In these characters, *Tomeurus* is like an oviparous atherinomorph. Eggs of viviparous atherinomorphs do not have filaments (Rosen and Bailey, 1963) and the zona pellucida is relatively thin (Grier, 1984); these characters are inferred to have been lost or reduced in the evolution of viviparity.

A relatively small proportion of viviparous poeciliids are known to be matrotrophic, i.e., embryos are nourished by the mother via a complex anatomical and physiological relationship. The typically zygoparous or embryoparous *Tomeurus*, when retaining embryos, is lecithotrophic, i.e., developing embryos are nourished by the egg yolk (Turner, 1937; Rosen and Bailey, 1963; Wourms, 1981; Reznick and Miles, 1989; Reznick et al., 2007b). Reversal from matrotrophy to lecithotrophy is considered complex as it would involve the loss of, or failure to express, modifications for embryo retention including placentation (Reznick and Miles, 1989; Wourms and Lombardi, 1992).

Few details are available on nourishment of *Cnesterodon* embryos or those of close relatives. Superfetation, females carrying more than one brood, was reported in another member of the tribe Cnesterodontini, *Phalloptychus januarius* (Hensel, 1868) by Turner (1937), following Stoye (1935), suggesting that *Cnesterodon* may also be matrotrophic (see Reznick and Miles, 1989; Reznick et al., 2007b).

Tomeurus shares reproductive characters with both oviparous and viviparous taxa as demonstrated by a comparison of these characters among select atherinomorphs representing each of the three orders and a variety of reproductive modes (Table 1). Spermatozeugma morphology is considered here a homology or synapomorphy that corroborates the classification of *Tomeurus* as a member of the monophyletic poeciliid subfamily Poeciliinae *sensu* Parenti (1981). Egg morphology and efferent duct secretory products are like those of oviparous atherinomorphs and are hypothesized to be plesiomorphic characters. Taken together, these characters support the phylogenetic hypothesis that *Tomeurus* is sister to viviparous poeciliines (Lucinda and Reis, 2005), with livebearing evolving once in the family Poeciliidae. Other hypotheses, that *Tomeurus* is sister to *Cnesterodon* (Ghedotti, 2000), or that *Tomeurus* is derived relative to *Xenodexia* (Hrbek et al., 2007), require reversal from viviparity to oviparity or independent origins of viviparity (see below). Also, the hypothesis, that *Xenodexia* is sister to all other members of the subfamily Poeciliinae *sensu* Parenti (1981) may reflect its being a phylogenetically long-branch (Reznick et al., 2007a:84; see also Hrbek et al., 2007). *Xenodexia* was hypothesized to be a member of a group of viviparous poeciliid genera that also includes *Poecilia*, *Limia*, *Pamphorichthys*, and *Micropoecilia*, in the morphological analysis of Lucinda and Reis (2005).

The sister group relationship of *Tomeurus* and *Cnesterodon* and the relationship of *Xenodexia* as sister to *Tomeurus* and the remaining viviparous poeciliids were both interpreted as evidence for secondary loss of livebearing in *Tomeurus* (Ghedotti, 2000; Reznick et al., 2007). Reversal of livebearing has been considered improbable by many (e.g., Blackburn, 2005): reversal from viviparity to oviparity would involve the loss of, or failure to express, complex morphological, physiological, and behavioral characteristics related to embryo retention and maternal provisioning (Mank and Avise, 2006). The alternative, that livebearing had independent origins, is considered more likely (Blackburn, 2005). We postulate one origin of viviparity in poeciliids on the basis of parsimony and do not speculate on the likelihood of a loss of viviparity.

Relaxing the traditional constraint that viviparity had evolved just once in the atherinomorph order Cyprinodontiformes meant the estimated number of origins of viviparity in that order increased from one to three (Parenti, 1981). There is a high number of oviparous–viviparous sister group pairs throughout vertebrates: a viviparous taxon may be closely related to an oviparous taxon, despite their divergent life-history patterns (Blackburn, 2005). Viviparity is not rare. Viviparity is broadly distributed among vertebrate lineages and estimated to have originated on at least 132 occasions in vertebrates (Blackburn, 1992).

Reproductive morphology and biology are rich sources of data for phylogenetic inference among atherinomorphs and other teleost fishes (Meisner, 2001; Parenti and Grier, 2004; Parenti, 2005, and references herein). Within teleosts, as far as known, viviparity is found only among the Neoteleostei (Nelson, 2006:206–207), with little postulated explanation or correlation for this phylogenetic constraint. Continued surveys are needed to understand the distribution of a broad range of characters in many taxa to avoid overgeneralization or oversimplification about the evolution of reproductive systems (e.g., Wourms and Lombardi, 1992). Development of the testis, form and development of sperm bundles, and relationship between embryo and mother are just three areas where continued research is expected to produce new data to test phylogenetic hypotheses as well as further understand the evolution of reproductive modes in fishes.

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