

Fecundity, the Germinal Epithelium, and Folliculogenesis in Viviparous Fishes

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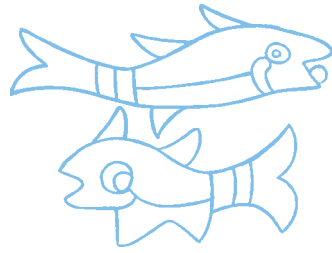
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Abstract

Reproduction in viviparous fish, and fish that practice internal fertilization but lay fertilized eggs, is shown to have evolved independently in several taxa based on the morphology of sperm packages, either spermatozeugmata or spermatophores. However, the origin of germ cells, both male and female, is from germinal epithelia that are apparently unchanged in spite of great diversity in gonad morphology. An exception is the Atherinomorpha where females have a typical ovarian germinal epithelium, but in males an epithelioid association between Sertoli cells and germ cells occurs because all of the criteria that define an epithelium are not met. Adaptations for internal fertilization and viviparity arise from new functions of the cells that are common in all fish, not the participation of new cell types. Based upon the restricted distribution of spermatogonia within testicular lobules in the Atherinomorpha, there is no support for relationships between them and the viviparous surfperches or mullet where spermatogonia may occur along the walls of the lobules. As a reproductive mode, viviparity results in greatly reduced fecundity compared to fish whose reproductive mode is external fertilization and scattering of eggs. It appears that concepts used in fisheries biology can be adapted to better understand viviparity.

Resumen

Se ha demostrado, con base en la morfología del empaquetamiento de los espermatozoides, tanto en spermatozeugmata como en espermátóforos, que la reproducción en peces vivíparos, y en peces que presentan fertilización interna pero que depositan los huevos fertilizados, ha ocurrido independientemente en diversos taxa. Sin embargo, el origen de las células germinales masculinas y femeninas, es el epitelio germinal que, aparentemente, no ha cambiado a pesar de la gran diversidad de la morfología de las gónadas. Una excepción son los Atherinomorpha, en los cuales las hembras muestran un epitelio germinal ovárico típico, pero en los machos ocurre una asociación epitelioides entre las células de Sertoli y las germinales, debido a que no se reúnen todos los caracteres que definen a un epitelio. Las adaptaciones para la fertilización interna y la viviparidad se originaron, no por la ocurrencia de nuevos tipos celulares, sino por nuevas funciones de las células presentes en todos los peces. Con base en la distribución restringida de las espermatogonias dentro de lóbulos testiculares en Atherinomorpha, no se puede establecer relación entre ellos y embiotócidos o lisas en los cuales las espermatogonias pueden localizarse a lo largo de las paredes de los lóbulos. En la viviparidad, como modo reproductivo, ocurre una gran reducción de la fertilidad, comparada con la que presentan los peces que muestran modo reproductivo con fertilización y oviposición externas. Parece que los conceptos utilizados en biología de peces deben adecuarse para una mejor comprensión de la viviparidad.



Introduction

The Teleostei is a diverse clade of aquatic vertebrates that has evolved varied modes of reproduction. Most teleost fish scatter gametes into the external, aqueous environment. The embryo develops within an egg in surroundings that may be considered hostile towards its survival. Few eggs, larvae, or juveniles survive to become reproductive adults.

The number of eggs shed at a single spawning is termed “batch fecundity.” In their classic review, “Modes of Reproduction in Fishes,” Breder and Rosen (1966) reported batch fecundity in various fish species in an anecdotal fashion, common at that time. Since then, the measurement of batch fecundity has become an integral part of life history data used in fisheries science, inspired by the pivotal work of Hunter and colleagues (Hunter et al., 1985; Hunter and Macewicz, 1985). Egg-scattering fish produce enormous numbers of eggs at a single spawning event (batch fecundity), spawn multiple times within a spawning season (indeterminate annual batch fecundity), or spawn once per year (determinate annual batch fecundity). The estimation of batch fecundity, its relationship to indeterminate or determinate batch fecundities annually and within a spawning season, establishes research imperatives in fisheries science and documents a successful mode of reproduction (the scattering of enormous numbers of eggs into the environment with extremely low survivorship) that is typical for teleosts (*viz.* Breder and Rosen, 1966).

Viviparity, or internal fertilization coupled with giving birth to live young, is the mode of

reproduction for a relatively small number of teleosts (*viz.* Breder and Rosen, 1966). Approximately 3% of all teleost species are known to be viviparous (Callard and Ho, 1987; Wourms, 2004). Viviparity has evolved independently in several teleost clades (*viz.* Parenti, 1981; Lydeard, 1993). The concepts of fecundity as used in fisheries science have never been applied to the study of reproduction in viviparous fishes. As fisheries concepts are applied to commercial fisheries, so too might they be applied to the management and protection of viviparous fish populations, providing the means for documenting population status and developing management plans for species conservation.

Viviparous fishes have a much lower fecundity, by orders of magnitude, than fish that produce eggs (Table 1A,B). The enormous fecundity in fish that scatter eggs, however, is diminished when compared to viviparous species that have evolved unique adaptations to ensure internal fertilization, gestation, and birth. Our principal goal is to compare these two reproductive modes in fishes, egg-scattering and viviparity, within a framework of homology and analogy to understand the basis of reproductive diversity and to gain further insight into the evolution of morphological diversity among fishes. We also document some of the similarities and differences in gonad morphology between viviparous fishes and those with external fertilization and assimilate this information into a structural framework to interpret current knowledge and to guide future research.

Table 1.

Fecundity in Fish: **A. Saltwater Fish that Scatter Eggs.**
B. *Danio rerio* and Viviparous Fish.

Table 1A.

Family <i>Species</i>	Total Length (cm)	Spawning Frequency	Batch Fecundity	Annual Egg Production	Location	Reference
Labridae <i>Tautoga onitis</i>	26-52	1.2 days	2800 to 181,200	290,000 to 10,510,000	Chesapeake Bay Virginia, USA	White et al. 2002
Mugilidae <i>Mugil cephalus</i>	33-60	1 per annum	213,000 to 3,890,000	213,000 to 3,890,000	South Carolina	McDonough et al. 2003
Sciaenidae <i>Cynoscion nebulosus</i>		4.4 days	145,452 to 529,976	3.2 to 17.6 million	South Carolina USA	Roumillat & Brouwer, 2002
<i>Micropogonias furnieri</i>	40	3-4 days	216,000 in Nov 96,900 in Feb.	3.3 to 7.3 million	Rio de la Plata Argentina	Macchi et al. 2002

TABLE 1B.

Family <i>Species</i>	Standard Length (mm)	Average # Oocytes	Average # Embryos	Range	Location	Reference
Cyprinidae <i>Danio rerio</i>	33-34	668 +/- 277SE	NA	257 to 1115	Aquarium fish	unpublished
Poeciliidae <i>Gambusia affinis</i>	50	94.3 +/- 17	NA	56 to 113	Aquarium fish	unpublished
Poeciliidae <i>Poecilia reticulata</i>	30-31	NA	17.2+/-7.02	10 to 33	Aquarium fish	unpublished
Goodeidae <i>Xenotoca eiseni</i>	42-44	NA	25.5+/-3.43	19 to 32	Aquarium fish	unpublished

Why Viviparity?

Estimated batch fecundity (BF) is presented for four species of marine, perciform fish and, by actual egg count, one species of freshwater cyprinid, popular as an aquarium fish (Table 1A). These examples of fecundity are contrasted against the much lower brood fecundity of viviparous fishes in the cyprinodontiform atherinomorph families Poeciliidae and Goodeidae (Table 1B). The association between a brood of young, born to a viviparous fish, and batch fecundity, as the concept was developed for fish that scatter eggs (Hunter et al.,

1985; Hunter and Macewicz, 1985), has not been established previously. Herein, we establish the concept that viviparity is a special kind of BF, specifically “indeterminate batch fecundity” because viviparous fish give birth to multiple broods of young. In viviparous fishes, BF is the actual number of intra-ovarian young that are counted upon dissection of, or the number of young born to, a female. BF in a viviparous fish can be determined with accuracy in contrast to estimating the large numbers of eggs that are produced during a spawning event in most externally-fertilizing species (Table 1A).

Both fisheries and captive spawning data of common snook, *Centropomus undecimalis* (family Centropomidae, order Perciformes; Nelson, 1994), an inshore, marine fish that is common in passes and around mangrove islands in the tropics and subtropics (Taylor et al., 1998), have established that the spawning frequency is every 1.6 days during a protracted spawning season that lasts nearly four months, beginning in May and ending in September in Florida, USA (Taylor et al., 1998; Grier and Taylor, 1998). Moderate-sized females average an estimated 280,000 eggs per spawn when induced to ovulate using gonadotropin-releasing hormones (Neidig et al., 2000). There are no data on the BF of large females that might be expected to produce over a million eggs per spawning event. As a rough estimate, a single common snook female could produce 7 to 8 x 10⁹ eggs during her reproductive lifetime. If larvae from only two of the eggs grow to become reproductive, spawning adults, then steady-state population dynamics should be maintained. The startling conclusion from this simple example is that survival from egg to adult is abysmally low. The number of eggs that survive to grow into reproductive adults approximates 0.000,000,004% over the lifetime egg production of a female common snook.

Given the estimated annual egg production of marine fish, and BF of one freshwater fish species (Table 1A,B), an inference is the extremely low survival of eggs, larvae that hatch, and growing juveniles for egg-scattering fishes. The probability of a “scattered fish egg” hatching and the larva growing to a spawning adult is probably not significantly different from zero, but that low probability succeeds at the population level, guaranteeing the survival of numerous species because of their enormous fecundity.

In contrast to the scattering of eggs into the environment or carrying embryos to birth, the reproductive mode of a small number of teleost taxa involves internal fertilization and the laying of fertilized eggs, as in the atherinomorphs *Horaichthys setnai* (see Kulkarni, 1940; Grier, 1984), *Labidesthes sicculus* (see Grier et al., 1990), and in select ostariophysans (Burns and Weitzman, 2004). A fine distinction between internal fertilization and internal gametic association needs to be recognized. Internal gametic association is when sperm and eggs associate in the ovarian lumen, but eggs are not fertilized until they are laid and sperm are activated by water (Burns and Weitzman, 2004). Internal gametic

association may be suspected when it is known that an isolated female may lay eggs that subsequently develop, yet developing eggs are not found in the ovarian lumen where sperm are present. In the atherinopsid, *Labidesthes sicculus*, internal fertilization is known to occur because eggs with developing embryos were observed in the ovarian lumen (Grier, et al., 1990). BF estimates and spawning frequencies are not available for fishes with these modes of reproduction. BF may not be much different from those of fish that scatter eggs, after accounting for size variation in spawning fish, however.

In viviparous fish, BF might be expressed as the number of young in relationship to either the standard length (SL), the total length (TL) (expressed in mm or cm), or the weight (gms) of the female times 100, as with the gonadosomatic index (GSI). Insofar as the gonad might represent 35% or more of the total weight of a viviparous female prior to giving birth, but a much lower percentage after birth or early in embryonic development (Grier, unreported), gonad weight should be subtracted from total weight if BF is based upon female weight. The measurement of BF as a ratio (based on length or weight) might allow intraspecific fecundity estimates within a population or interspecific fecundity estimates based on real species differences:

$$\text{BF} = \text{Number of young} \times 100$$

$$\text{or BF} = \frac{\text{Number of young}}{\text{Female weight}} \times 100$$

SL or TL Total weight-Gonad weight

There are no data on BF in viviparous fish from natural populations as they may relate to changes during an annual reproductive cycle, by size, by age, or by way of comparisons to egg production in fish that have external fertilization. The data presented in Table 1B come from data on 25 captive fish of each species, at the specific sizes indicated. The poeciliids, *Poecilia reticulata*, the Guppy, and *Gambusia affinis*, the Western Mosquitofish, were maintained in aquaria on a long photoperiod (16 hours of light per day) at 26 to 28 °C. The goodeid, *Xenotoca eiseni*, was maintained in an outdoor, plastic-lined pool under conditions of natural day length and temperatures prevailing during the spring and summer in Florida. The *Xenotoca* data are derived from a group of one-year old fish in a size range of 42-44 mm SL. Fecundity information for other goodeids include *Chapalichthys encaustus* in which there were sel-

dom more than ten embryos, and *Goodea atripinnis* in which a range of 15-60, approximately 2 cm long, embryos were noted (Meyer et al., 1985), but sample size was not reported. BF from Table 1A can be estimated based on standard length. The annual production of young would be analogous to indeterminate BF in egg-scattering fish, provided that the average interval between broods is known. Birth frequency in viviparous fish is probably significantly correlated with temperature, and may vary during a reproductive season. By the application of common parameters used in fisheries science, important aspects of life history could be ascertained, and meaningful comparisons might be made pertaining to viviparous fish populations and species.

The data for large female Western Mosquitofish (Table 1B) can be used as a rough indicator to estimate BF and annual production of young for this size class maintained in aquaria. Reproduction in wild *G. affinis* extends over six to seven months in Florida, from February or March through September, and the interval between broods is 21-24 days at 26 to 28 °C (Grier, unreported). A female would be expected to produce approximately nine broods during a spawning season. In these large female *G. affinis* (Table 1B), the number of young produced during a spawning season would range approximately be-

tween 400 and 800. BF in the smaller Guppy is even lower when compared to the production eggs in fish species with external fertilization (Table 1A, B). Nevertheless, this does not preclude high fecundity in guppies, long known by aquarists as the “millions fish” (Sterba, 1959; Hoedeman, 1974).

Many of the Goodeidae are listed as threatened or endangered (Domínguez-Domínguez et al. 2004). Similar information on brood intervals and BF in natural populations of goodeids, or data generated from captive stocks, might be useful for developing species management plans for threatened or endangered viviparous fishes. Viviparity confers some obvious survival advantages over external fertilization and egg scattering, even though there is a greatly reduced batch fecundity and annual young production.

Gonad Morphology

Males:

To introduce sperm into the female reproductive tract, with or without internal fertilization followed by the laying of fertilized eggs or viviparity, it is not efficient for males to release free sperm into the water, as there is little expectation that sperm will enter the female reproductive tract. Once exposed to an external milieu, sperm become activated and have a rather short life span, as demonstrated for trout (Billard et al., 1987). Without exception, fish that practice internal fertilization have evolved intricate morphological adaptations for the transfer of sperm to the female reproductive tract where sperm may remain viable for an extended time period. The array of morphological adaptations include: [1] sperm transfer to the female reproductive tract by highly modified male anal fins (Greven, 2004; Meisner, 2004), [2] via a genital papilla (Grier et al., 1990), [3] neuronal modification coupled with evolution of a coordinated behavior (Greven, 2004; Rosa-Molinar, 2004), [4] sperm packaging in an acellular capsule or spermatophore, as in *Horaichthys setnai* (Kulkarni, 1940; Grier, 1993), and [5] production of naked sperm bundles or spermatozeugma (singular) or spermatozeugmata (plural) (*vide infra*; Grier, 1993). In *Anableps anableps* and *Jenynsia lineata* spermatozeugmata are not formed, but free sperm are transferred into the female reproductive tract through tubular gonopodia (Grier et al., 1981). In the halfbeak genus *Zenarchopterus*, free sperm are released into the testicular efferent ducts during spermiation where they associate

Fig. 1. A lobule terminus in the testis of the striped mullet, *Mugil cephalus*, is outlined by pink-staining interstitial cells. There is a continuous GE, illustrated by a continuous distribution of spermatogonia (SG) and spermatocysts, each containing spermatocytes (SC), spermatids (ST), or sperm (SP), gamete development is synchronous within spermatocysts. Sperm are also present in the lobule lumen (SP*). The clear lines between neighboring spermatocysts are Sertoli cell processes (SE), defining the borders of the spermatocysts. Bar = 50 µm

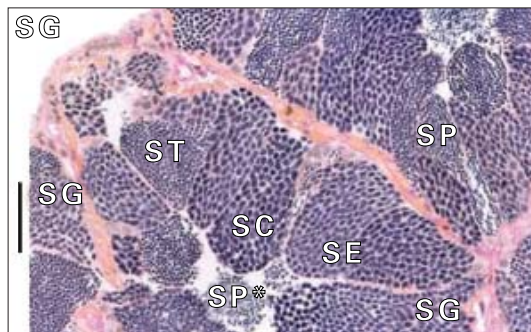
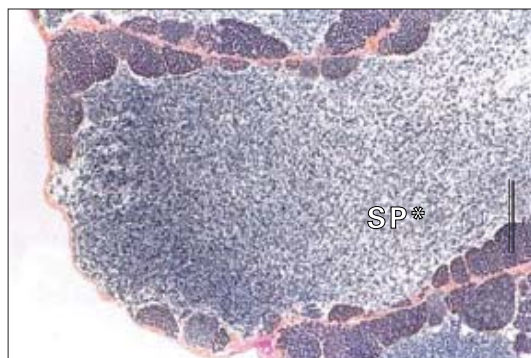


Fig. 2. During late maturation, a discontinuous GE is observed at the terminus of the lobule in the testis of the striped mullet, *Mugil cephalus*, and spermatocysts are absent (arrows) from part of the GE; the lumen of the lobule is engorged with sperm (SP*) in this fish that spawns once per year. Bar = 100 µm



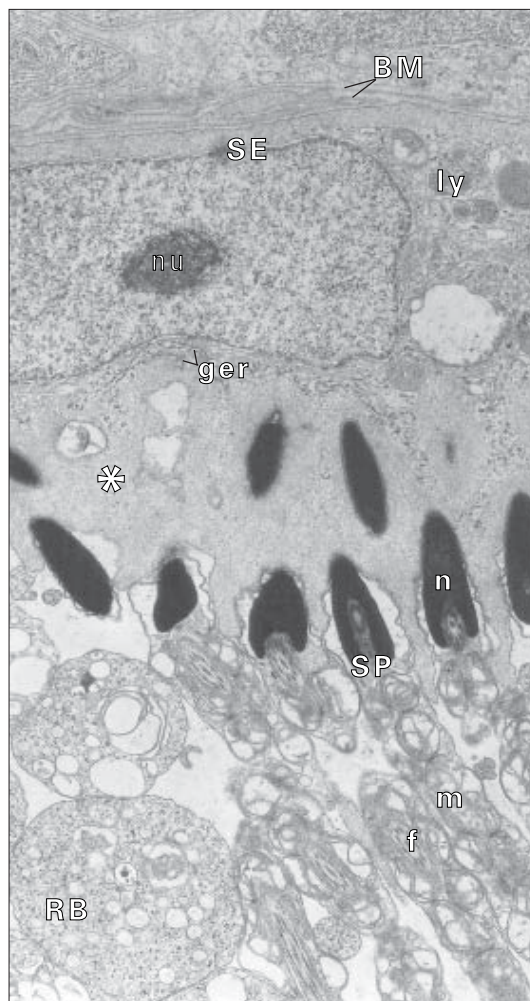
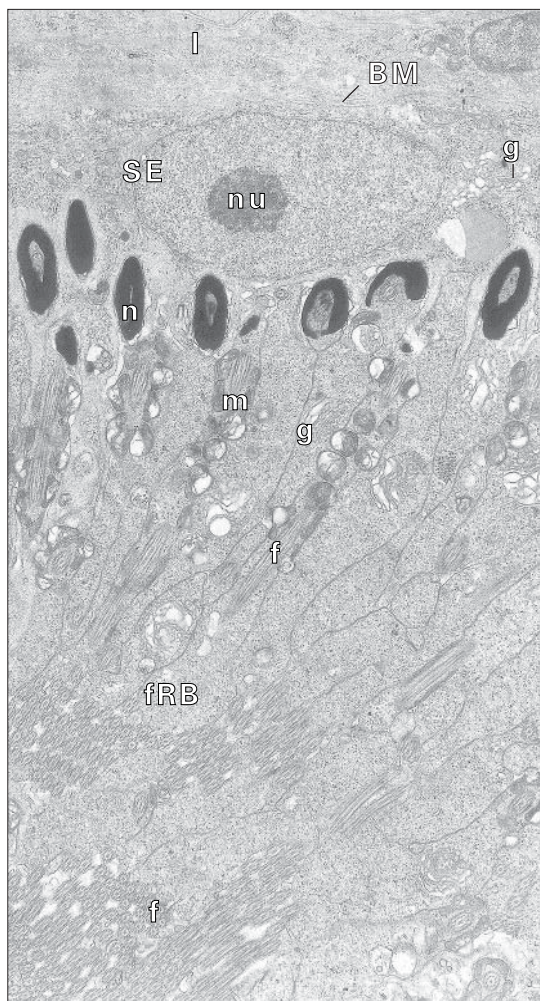


Fig. 3. The periphery of a spermatocyst in the testis of the poeciliid, *Xiphophorus helleri*. Spermatids, their nuclei with condensed chromatin (n), are associated with Sertoli cells (SE). Each spermatid is forming a residual body (fRB) in which the trailing cytoplasm has a Golgi (g) apparatus, mitochondria (m), in what will be the midpiece, and trailing flagella (f). Sertoli cells have an oval nucleus with a well-developed nucleolus (nu), and a Golgi apparatus (g). The spermatocyst, within the germinal compartment of the testis, is separated from the interstitium by a basement membrane (BM). Bar = XX μ m

Fig. 4. The periphery of a spermatocyst in the testis of the poeciliid, *Xiphophorus helleri*. Residual bodies (RB) are in the lumen of the spermatocyst. Sperm (SP) nuclei (n) are embedded in a region of Sertoli cell (SE) cytoplasm that is devoid of organelles such as granular endoplasmic reticulum (ger) and lysosomes (ly). The sperm midpiece of composed of a flagellum (f) surrounded by mitochondria (m). The Sertoli cell nucleus has a well-developed nucleolus (nu). At this stage of sperm maturation, the basement membrane is becoming multi-layered. Bar = XX μ m

and form elongated spermatozeugmata (Fig. 11; Grier and Collette, 1987). In *Zenarchopterus dispar*, the morphology of spermatozeugmata is not determined by the associations of Sertoli cells and germ cells that form within the spermatocysts, as in the Poeciliidae and the Goodeidae (*vide infra*). It is to be expected that fish practicing internal fertilization will use different mechanisms to package sperm for transfer to the female reproductive system, more of which likely remain to be discovered.

Compartmentalization characterizes the vertebrate testis, whether or not the reproductive mode is viviparity. Gametes are produced within the germinal compartment. Within the interstitial compartment are found hormone-secreting Leydig cells, myoid cells primarily coursing over the surface of the germinal compartments and separated from them by a basement membrane (Grier 1993), blood supply, and immune system cells, most notably eosinophilic granulocytes in fishes. Initial attempts to identify ho-

mologous cell types in some fish testes led to erroneous interpretations and confusion. For a time, it was believed that some fish (*Esox lucius* and *Salmo salar* [see Marshall and Lofts, 1956]) had lobule boundary cells as Leydig cell homologs. This misinterpretation resulted from use of techniques with inherent low resolution—Sudan staining to demonstrate lipids in frozen sections. Upon ultrastructural examination, it was revealed that the lipids in the testes of *Esox lucius* were not within the cytoplasm of a Leydig cell homolog in the interstitial compartment of the testis. The lipids were in the cytoplasm of Sertoli cells located within the germinal compartment. In *Esox niger*, however, lipids were not observed within Sertoli cells. The presence or absence of lipids in the testes of *Esox* species underscores reproductive differences within the genus, and also reflects on reproductive diversity of other fish. It is not possible for an interstitial cell type, the Leydig cell, to be homologous with a cell type, the Sertoli cell, in the germinal com-

Fig. 5. The periphery of a spermatocyst in the testis of the poeciliid, *Xiphophorus helleri*, in which phagocytosis and digestion of residual bodies (RB) are illustrated. Sertoli cell processes are observed to be engulfing one residual body (asterisk) while a second residual body (RB) is within the Sertoli cell cytoplasm. Other phagocytized residual bodies have become digestive vacuoles (DV), all with electron dense breakdown products (arrow heads). The Sertoli cell has a well-developed nucleus with a single nucleolus (nu) and cytoplasmic lysosomes (ly). One sperm is also observed to be in the process of degradation, surrounded by membranous lamellae (l). Sperm nuclei (n) are embedded in the cytoplasm of the Sertoli cell (SE). Their midpieces (mp) and flagella (f) extend into and occupy the center of the spermatocyst. At this stage of development, just before spermiation, the basement membrane has become multi-layered. Bar = XX μm

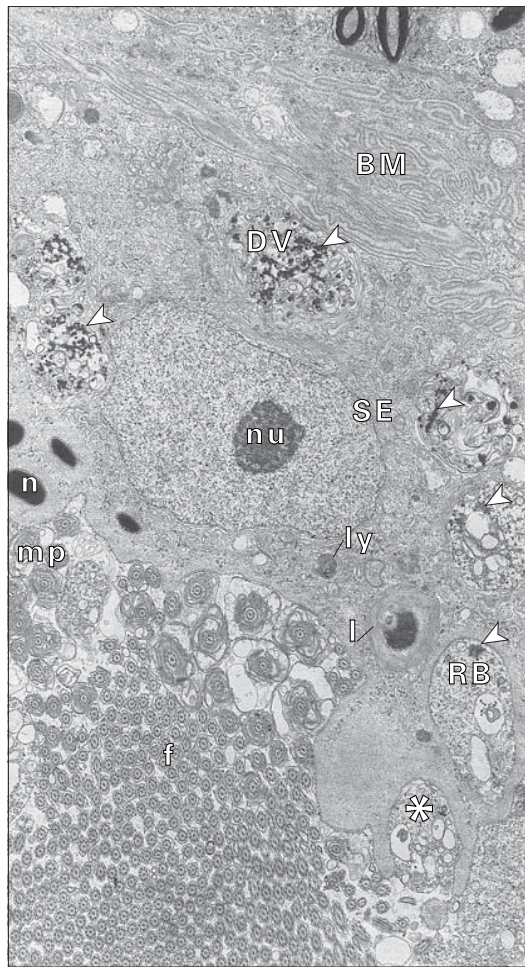
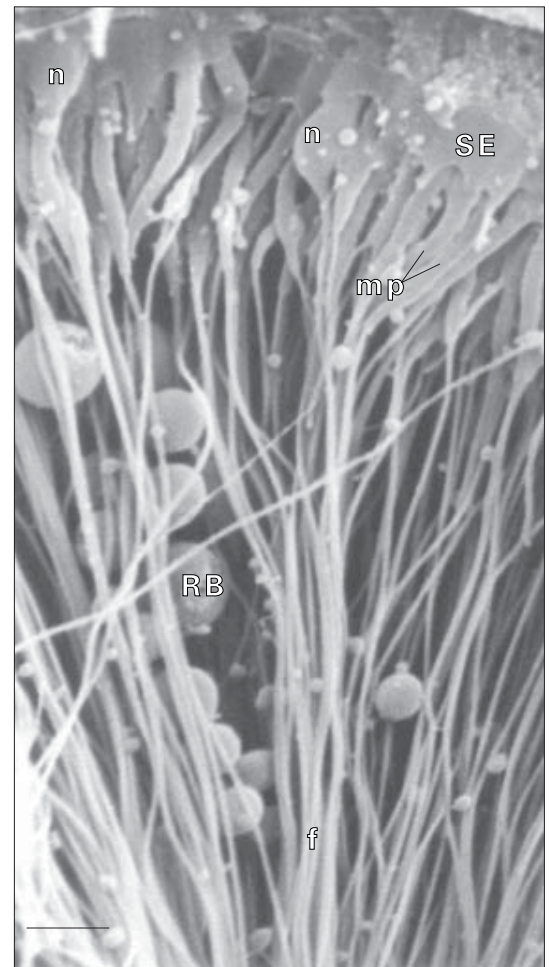


Fig. 6. The periphery of a spermatocyst from the testis of the poeciliid, *Poecilia reticulata*. Sertoli cell processes (SE) are observed as they envelope sperm nuclei (n). These processes are those that are devoid of organelles in the previous micrographs. Sperm midpieces (mp) and flagella (f) extend into the spermatocyst. Spherical residual bodies (RB) are observed among the flagella. Bar = 2 μm



(Callard, 1991). Anastomosing tubular testes occur in basal teleosts. Higher teleosts possess lobular testes (Grier, 1993; Parenti and Grier, 2004) in which the germinal compartments end blindly at the testis surface (Figs. 1, 2). The phylogenetic transition between anastomosing tubular and lobular testes has not yet been clearly identified, although Parenti and Grier (2004) proposed the lobular testis as diagnostic of the Neoteleostei. So far as is known, viviparity occurs in an array of neoteleost taxa, all of which have lobular testes (Lydeard, 1993; Parenti and Grier, 2004).

A second character of the vertebrate testis is phagocytosis of residual bodies that are cast off by maturing spermatids, as in the poeciliids *Xiphophorus helleri* (Figs. 3-5) and *Poecilia latipinna* (Fig. 6), and the goodeid, *Xenotoca eiseni* (Fig. 7). Phagocytosis of residual bodies designates Sertoli cell homology between vertebrate taxa (Grier, 1993). Other characters that might denote homology of synthesis are androgen binding protein and formation of a blood

testis barrier, but sufficient comparative information is lacking.

The presence of a basement membrane is one of the defining characters of an epithelium, a germinal epithelium (GE) with both a somatic cell type(s) and a germ cell type(s). Both males and females have germinal epithelia that are separated from interstitial or stromal tissues, respectively, by the basement membrane. It is important to recognize basic, homologous structures and similar cell functions in gonad morphology as these can be used to establish a comparative nomenclature (Grier, 1993) that underlies the “unifying concept,” (Grier, 2000) wherein the nomenclature and definitions applied to homologous structures in vertebrate gonads are identical.

Spermatozeugmata and spermatophores in internally fertilizing fishes form because of modified associations between Sertoli cells and germ cells that do not occur in externally fertilizing fishes. Their formation does not include the evolution of new cell types, but altered func-

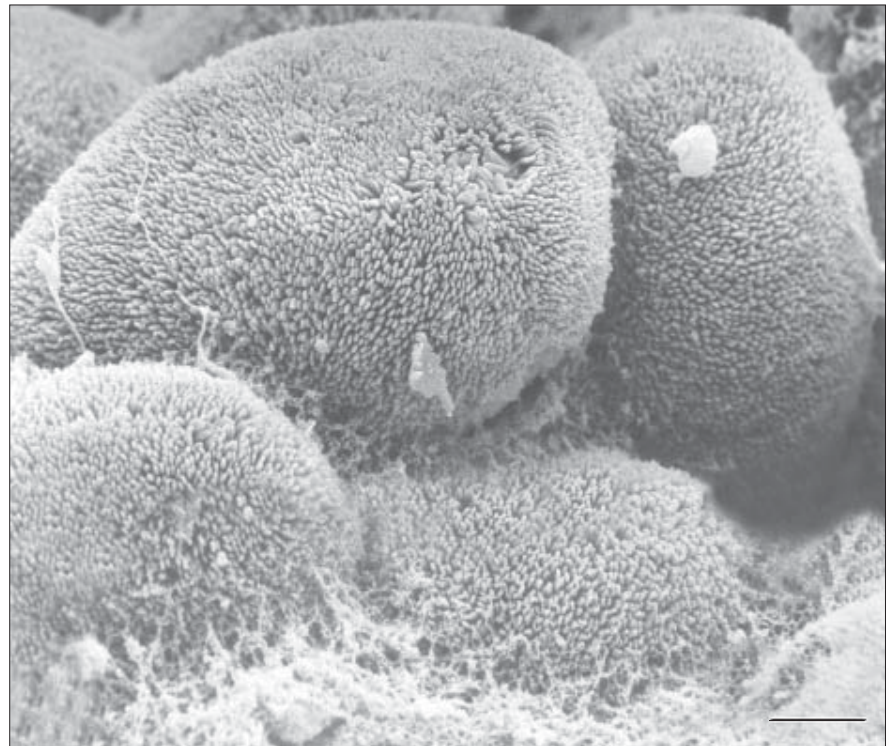
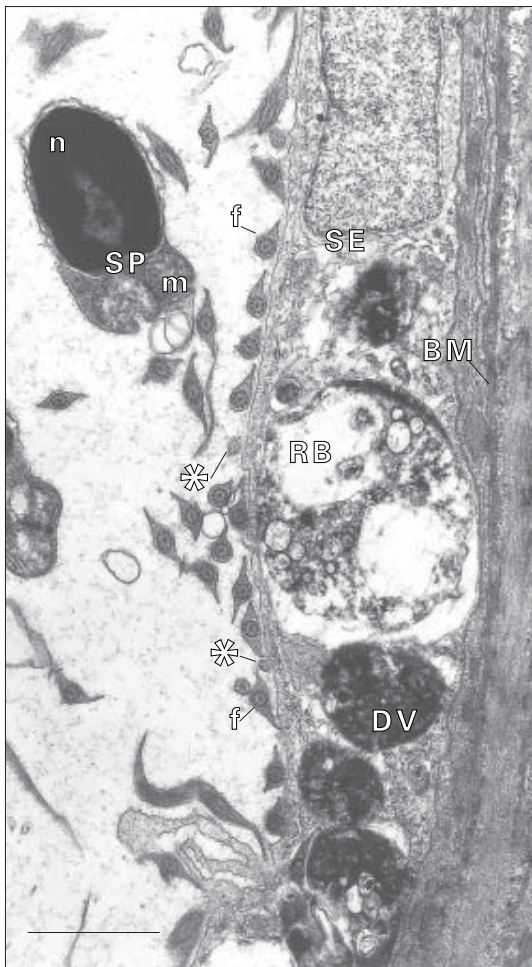


Fig. 7. The periphery of a spermatocyst from the goodeid, *Xenotoca eiseni*. A phagocytized residual body (RB) and digestive vacuoles (DV) are visible within Sertoli cell (SE) cytoplasm. A basement membrane (BM) separates the Sertoli cell from the interstitium. Within the spermatocyst, a sperm (SP) is visible and their flagella (f) associate, even at the distal ends (asterisks), with the Sertoli cell plasmalemma. Sperm nucleus (n), mitochondria (m) in the sperm midpiece. Bar = 2 μ m

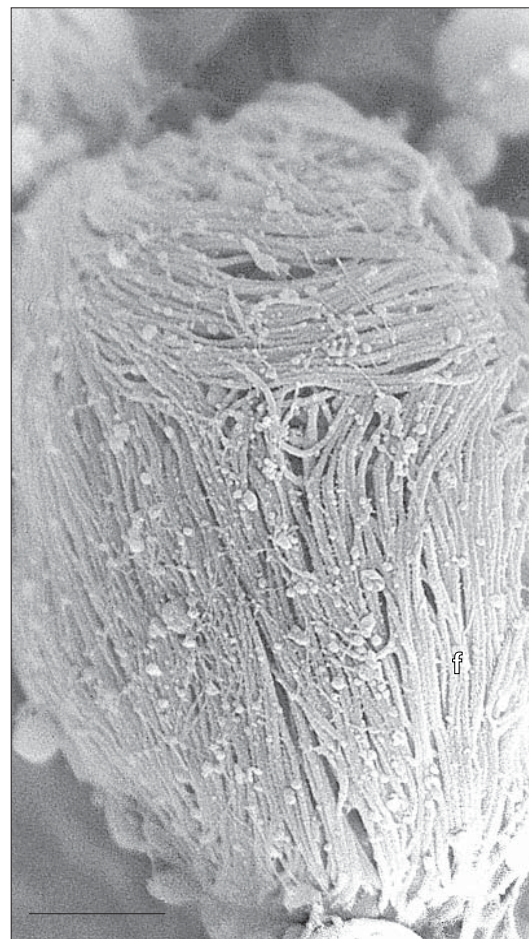


Fig. 8. Spermatozeugmata from the testis of the poeciliid, *Poecilia latipinna*, have a surface composed of sperm nuclei. Bar = 10 μ m

Fig. 9. Spermatozeugma from the testis of the goodeid, *Xenotoca eiseni*, have a surface composed of sperm flagella (f). Bar = 4 μ m

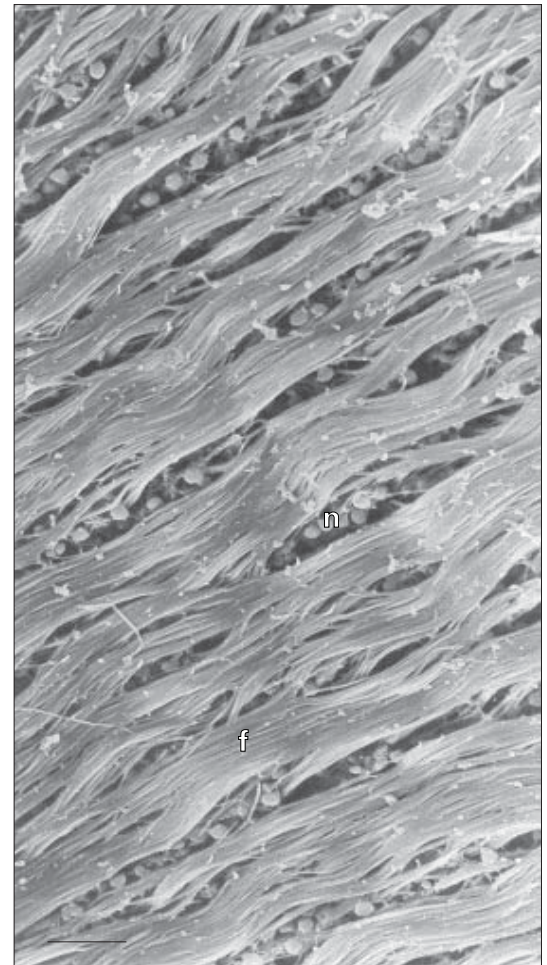
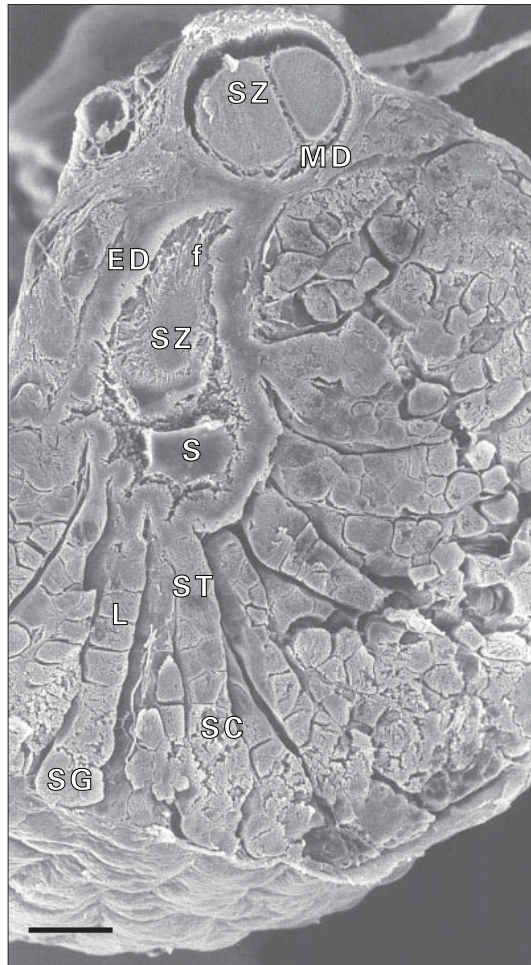
tion and morphology of the same cell types that are found in all fish testes. The associations between Sertoli and germ cells, correlated with sperm packaging, have evolved independently in different taxa. For example, in the Poeciliidae, elongating spermatid nuclei become associated with Sertoli cells (Figs. 3-5). Within the efferent and sperm ducts, spermatozeugmata are observed where their surface is composed of sperm nuclei (Fig. 8). In the Goodeidae (Fig. 7; Grier et al., 1981), spermatid flagella become associated with the Sertoli cells, and sperm flagella course over and form the surface of spermatozeugmata (Fig. 9). Both of these cyprinodontiform atherinomorph families (sensu Parenti, 1981) have evolved viviparity independently as reflected in the formation and morphology of spermatozeugmata, and as also reflected by intrafollicular (Poeciliidae) versus extrafollicular gestation (Goodeidae, Fig. 10), embryonic adaptations (Turner, 1933; 1937; 1940), and geographic isolation (Poeser, 2004; Contreras-McBeath, 2004).

Fig. 10.

The testis from the hemiramphid, *Zenarchopterus dispar*, showing the structure of lobules (L) in relationship to the efferent ducts (ED). As with all atherinomorphs, spermatogonia (SG) are restricted to the distal termini of the lobules that terminate blindly at the testis periphery. Extending proximally, spermatocysts containing spermatocytes (SC), and spermatids (ST) are observed. Free sperm are voided into the efferent ducts whereupon they coalesce to form spermatozeugmata (SZ), observed in the efferent and main (MD) ducts. Modified from Grier and Collette, 1987, used with permission from *Copeia*. Bar = 100 μ m

Fig. 11.

Spermatozeugma from the hemiramphid, *Zenarchopterus dispar* have a surface composed of parallel arrays of sperm flagella (f). Where these arrays separate, sperm nuclei (n) are observed. Bar = 10 μ m



Within the Atherinomorpha, internal fertilization, with or without viviparity, is inferred to have evolved independently within several other familial groups including: Hemiramphidae (Grier and Collette, 1987; Meisner, 2004), Adrianichthyidae (Kulkarni, 1940; Grier, 1984; Rosen and Parenti, 1981), Anablepidae (Grier et al., 1981), Poeciliidae genus *Tomeurus* (Rosen and Bailey, 1963), and Phallostethidae (Grier and Parenti, 1994), as reflected by dissimilar morphology. Within the developing spermatocysts of *Anableps anableps*, *Anableps dowi*, and *Jenynsia lineata* (Anablepidae), sperm associate with Sertoli cells in similar fashion to that observed in the Poeciliidae. Anablepids possess tubular gonopodia, however, which apparently obviates the need to package sperm. *Anableps dowi* is the only one of these three species noted above that forms “partial spermatozeugmata” (Grier et al., 1981), whereas the other two species release free spermatozoa into the efferent ducts. Associations between germ cells and Sertoli cells form, as in the poeciliids (Figs. 3-6).

Spermatozeugmata are not formed at spermiation, however, but individual sperm are released into the efferent ducts. Spermatozeugmata, similar to those observed in other poeciliids, are also observed in the testes of *Tomeurus gracilis* (Grier and Parenti, unreported).

Independent evolution of internal fertilization within the Atherinomorpha is also reflected in the halfbeak *Zenarchopterus* species (Grier and Collette, 1987) and the ricefish *Horaichthys setnai* (Kulkarni, 1940; Grier, 1984). In *Zenarchopterus dispar*, sperm are voided into the efferent ducts after which they begin to associate into, and form, elongated spermatozeugmata (Fig. 11) whose surface consists primarily of sperm flagella (Fig. 12). The morphology of spermatozeugmata varies somewhat between species within the genus, but they always form in a similar fashion, hypothesized to be a synapomorphy for the genus (Grier and Collette, 1987). In *Zenarchopterus dispar*, a secretory product is observed within the testis ducts (Fig. 11). The secretion appar-

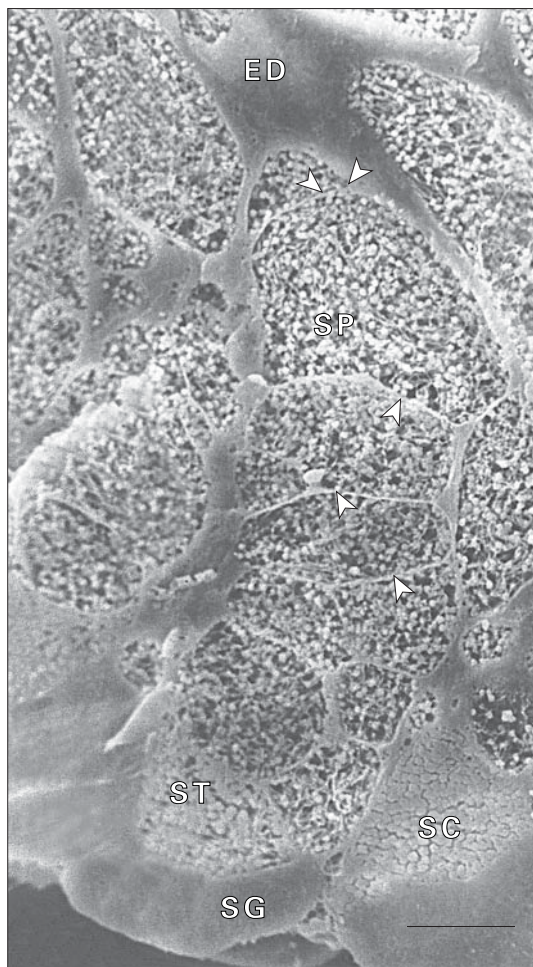
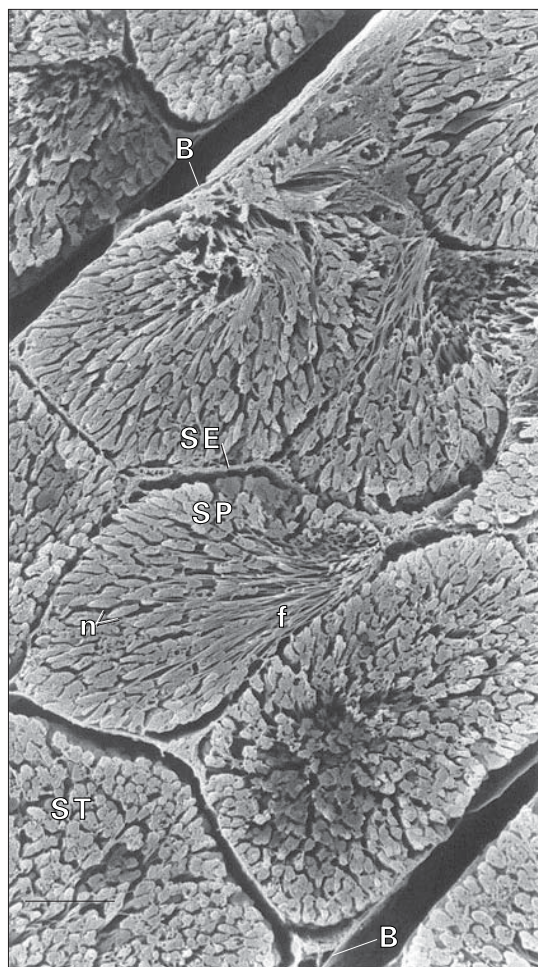


Fig. 12. In the “epithelioid” arrangement of Sertoli cells (SE) and germ cells, here spermatids (ST) and sperm (SP), spermatocysts extend across the width of a lobule. There is no lumen. The structure of spermatocysts, as being composed of synchronously-developing germ cells surrounded by thin Sertoli cell processes, is clearly evident in the testis of the hemiramphid, *Zenarchopterus dispar*. Boundary cells (B), sperm nuclei (n), sperm flagella (f), Bar = 10 μ m

Fig. 13. In the fundulid, *Fundulus grandis*, freeze fracture admirably illustrates Sertoli cell processes (arrow heads), encompassing single spermatocysts, which span the width of the lobule. Between the curved arrowheads, a Sertoli cell process does not quite span the width of the lobule, and spermiation, release of sperm into the efferent ducts (ED), is beginning. As in all atherinomorph testes, spermatogonia (SG) are restricted to the testis periphery, here being a homogeneous mass as the fracture technique only differentiates between cells or tissues and the intercellular space around them. Spermatocytes (SC), spermatids (ST), sperm (SP). Bar = 20 μ m

ently originates from the efferent duct cells that develop from hypertrophied Sertoli cells at spermiation as in other atherinomorphs (Grier, 1993). Again, histology indicates that Sertoli cells in the Atherinomorpha transform into efferent duct cells, becoming columnar and secretory. In the poeciliids, the efferent ducts are filled with a periodic acid Schiff-positive secretory product (Fig. 14) that permeates the spermatozeugmata. This secretion originates from efferent duct cells, as in *Poecilia latipinna* (van den Hurk and Barends, 1974) and *Brotula multibarbata* (Fig. 18b). In the Atherinomorpha, a special case of sperm packaging occurs in *H. setnai*, the only species in which true spermatophores are formed (Kulkarni, 1940). The spermatophore capsule is formed by the secretion that is produced by hypertrophied Sertoli cells (Figs. 15a, b, c; Grier, 1984). In *H. setnai*, the Sertoli cell secretion condenses around the sperm within a spermatocyst to form a spermatophore. The hypertrophy of Sertoli cells may be one more character that

supports atherinomorph monophyly, the most cogent being the restricted distribution of spermatogonia to the distal termini of the lobules (Parenti, 2004; Parenti and Grier, 2004).

The Sertoli and germ cells compose an epithelium, specifically a germinal epithelium (GE), in most taxa (Grier, 2000; Grier and Lo Nostro 2000). Association of the cells meets the criteria of an epithelium. Cells that compose the GE: 1) are connected laterally by cytoplasmic interdigitations, desmosomes, and tight junctions (Grier, 1993, 2000), 2) rest upon a basement membrane by histological definition (Figs. 3-5, 7), 3) border a body cavity (a lumen), and 4) are avascular.

A testicular GE occurs in most fish, including internally fertilizing fish (Figs. 17a, 18a), the exception being the Atherinomorpha where there is no lumen within lobules (Fig. 11,12; Parenti and Grier, 2004). Rather than a lumen, Sertoli cell processes, forming the borders of spermatocysts, “bridge” the width of lobules. Therefore, the third defining character of an

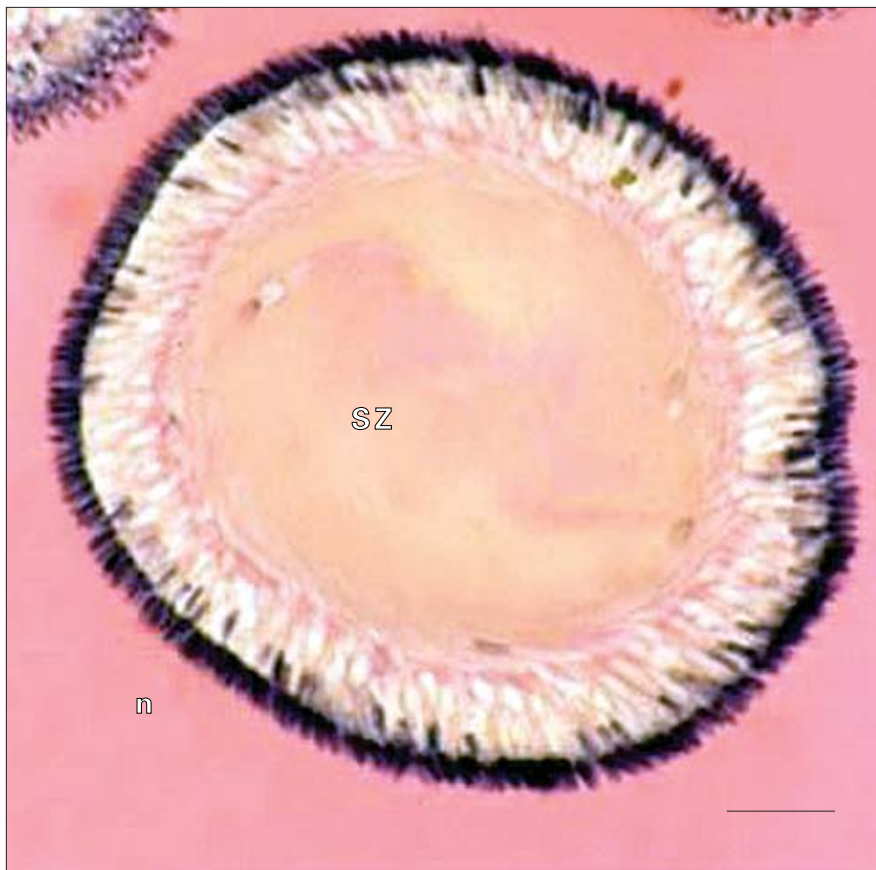


Fig. 14. A spermatozeugma (SZ) from the poeciliid, *Cnesterodon decemmaculatus*, is surrounded by periodic acid Schiff-positive (magenta) secretion that permeates into its center. Its circumference is composed of sperm nuclei (n). Bar = 10 μ m

epithelium, above, is not met. Without a central lumen, although satisfying the other criteria that define an epithelium, the Sertoli and germ cells in atherinomorph fish must be considered to have an epithelioid cellular association (Parenti and Grier, 2004). Hypothetically, a simple rearrangement in the association of Sertoli cells and germ cells, at the time when spermatocysts form, probably restricts spermatogonia to the distal termini of lobules in the Atherinomorpha as in both *Zenarchopterus* (Fig. 11) and in *Fundulus* (Fig. 12), Sertoli cell cytoplasm extends across the lobules even at the testis periphery, and no lumen forms.

The term “epithelioid” was first applied to the fish testis to describe annual gonad maturation classes in cobia, *Rachycentron canadum* (Brown-Peterson et al., 2002), a large, pelagic perciform fish in the family Rachycentridae (Nelson, 1994). During the regression class in cobia, cords of Sertoli and germ cells (spermatogonia) grow from the distal ends of the lobules, extending the lengths of lobules. A central lumen exists within the lobules, but not these developing cords of tissue. All of the criteria necessary to define an epithelium are not present,

i.e., the cells involved, the Sertoli and germ cells that will produce sperm, do not border upon a lumen. Prior to the initiation of meiosis in cobia, a lumen begins to develop within these cords and, thus, the initial epithelioid association of Sertoli and germ cells becomes a GE. An epithelioid arrangement of Sertoli and germ cells does not transform into a GE in the Atherinomorpha.

Spermatozeugmata are formed in some non-atherinomorph fish taxa. The Embiotocidae, or surfperches, comprise a family of some two-dozen species of viviparous fishes inhabiting the north Pacific coast of western North America, Japan and Korea (Nelson, 1994). It was suggested that surfperches share a restricted spermatogonial testis type (Grier et al., 1980) with the Atherinomorpha, a testis type not found in putative atherinomorph sister groups (Parenti and Grier, 2004). The purported homology of testis structure between the embiotocids and atherinomorphs is rejected upon examination of the maturation process, below. Unfortunately, specimens available to us precluded sufficient histology to describe testis maturation from a single species through an annual reproductive cycle. It was necessary to “reconstruct” a process of testicular maturation by examination of fixed material from different species in which males were in different reproductive condition.

During early maturation in the embiotocid *Cymatogaster aggregata* (Fig. 16a), the testis is atherinomorph-like: 1) spermatogonia are restricted to the distal termini of the lobules. 2) There is a distal to proximal distribution of earliest and progressively later stages of developing sperm. 3) Sertoli cell processes bridge the widths of lobules; there is an epithelioid association between Sertoli and germ cells. As testis maturation progresses, as in *Embiotoca jacksoni*, 1) spermatogonia are not observed at the distal termini of the lobules (Fig. 16b). 2) The progression of developing sperm, from earlier to later stages of maturation, also breaks down. Earlier stages of maturing sperm may occur both distally and proximally within lobules: the walls of lobules are lined with spermatocysts with later-developing stages randomly distributed with earlier stages of maturation. The depicted distribution of developing sperm (Fig. 16b) can only occur when spermatogonia are located along the walls of the lobules and do not enter meiosis simultaneously, quite unlike any known atherinomorph but typical of other neoteleosts

(following Parenti and Grier, 2004). 3) Sertoli cell processes are no longer observed to extend across the diameters of lobules. An epithelioid Sertoli and germ cell association has vanished from the lobules. 4) During spermiogenesis, sperm become organized as a spermatozeugma within a spermatocyst prior to being released into a developing central lumen within the lobule. In all atherinomorphs examined, spermatozeugmata are released into the testis ducts, and there is no continuous lumen within the lobule that connects to the efferent ducts. These are distinctive differences in testis morphology between the Atherinomorpha and other fishes (Figs. 17a,b, 18a) that achieve internal fertilization, that have only been described recently (Parenti and Grier, 2004).

By analogy, and as in cobia testis cycling during the annual reproductive season (Brown-Peterson et al., 2002), an epithelioid arrangement of Sertoli and germ cells becomes a GE as in the surfperches, a proposed example of convergent evolution or homoplasy. Our histology indicates that Sertoli cells in surfperches hypertrophy after spermiation and secrete a periodic acid Schiff-positive product, another inferred homoplasy as this also characterizes the Atherinomorpha. The periodic acid Schiff-positive secretion observed between spermatozeugmata in the efferent ducts in surfperches (Figs. 16d, e) also occurs in the Poeciliidae (Fig. 14), and in the ophidiiforms *Dinematichthys* sp. (USNM 338466; Fig. 17a,c) and *Brotula multibarbata* (USNM 214124; Fig. 18a). At the end of the surfperch reproductive season, there is a nearly total loss of germ cells from within lobules, as in the embiotocid *Phanerodon furcatus*. Occasionally, a spermatozeugma is observed within a spermatocyst, and the germ cells are scattered, as spermatogonia (Fig. 16c), along the wall of the lobules being interspersed with columnar, secretory Sertoli cells. The interpretation of the GE in *P. furcatus* is that it has become discontinuous, as originally observed and defined in *Centropomus undecimalis* during mid and particularly late maturation (Grier and Taylor, 1998). A discontinuous GE is a special case where, during maturation, a Sertoli cell epithelium occurs in which there are scattered germ cells (Grier and Taylor, 1998; Taylor et al., 1998). The surfperch testis has characteristics of the restricted type of the atherinomorphs. By examination of the maturation process through the year, however, its non-

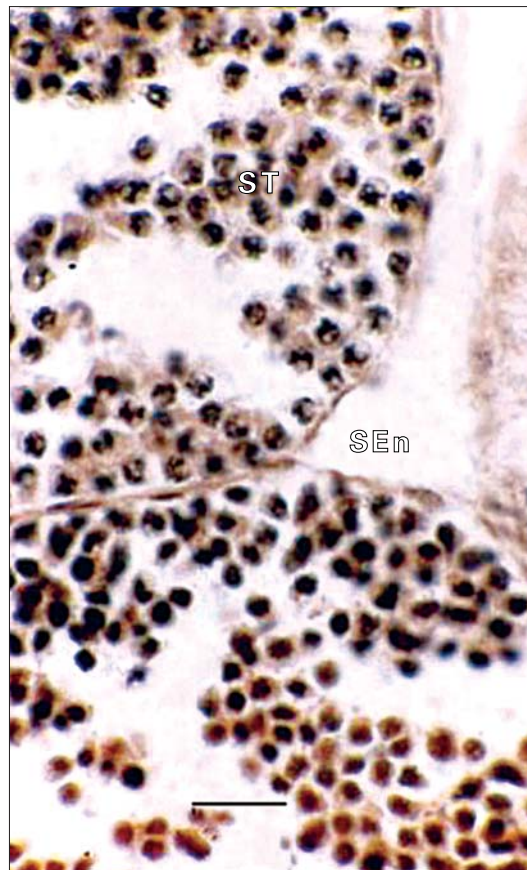
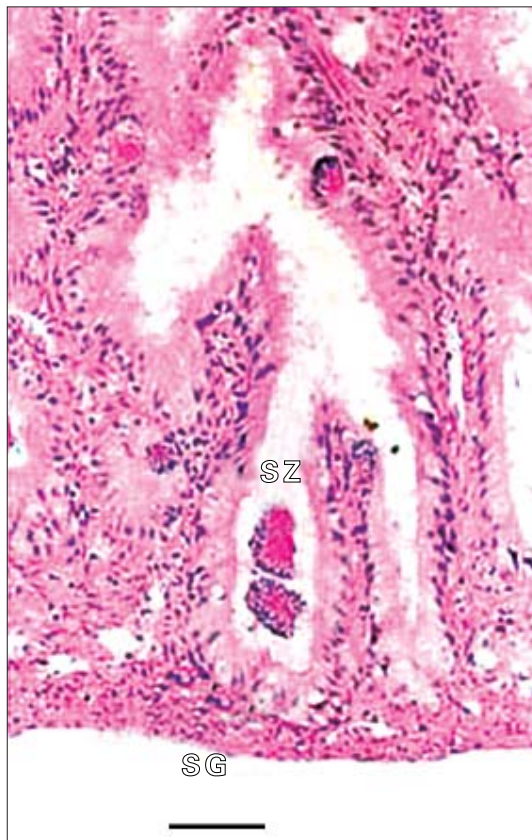
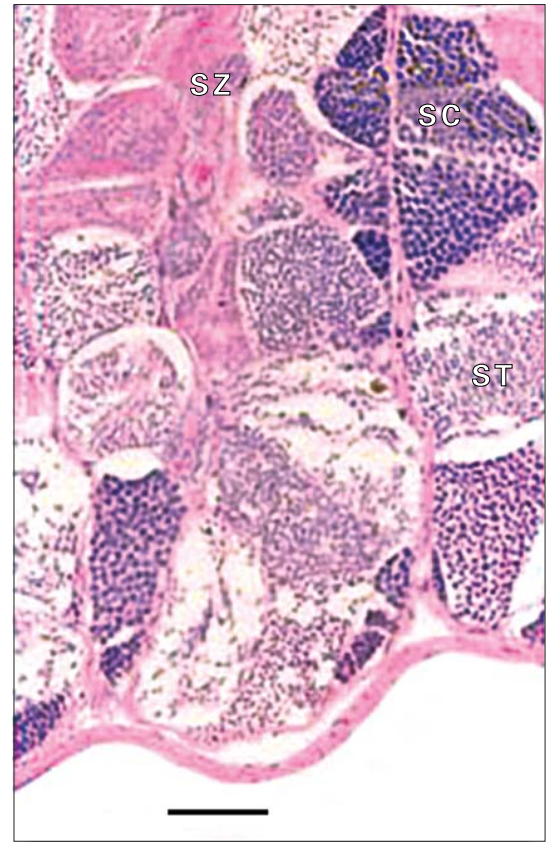
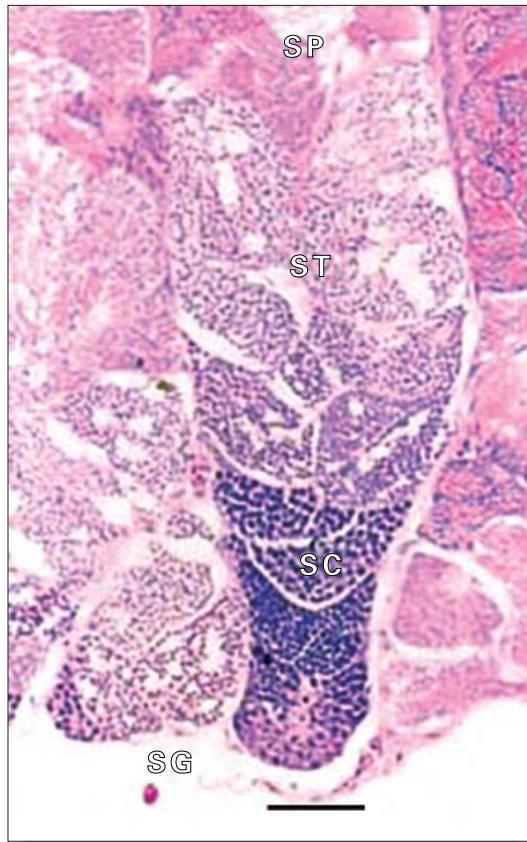


Fig. 15a-c. [a] Two spermatocysts containing spermatids from the testis of the adrianchthyid, *Horaichthys setnai*. The Sertoli cells have squamous nuclei (arrows). [b] During late spermiogenesis, the spermatids have condensed chromatin, and Sertoli cells have transformed into cuboidal cells with spherical nuclei. [c] Upon completion of spermiogenesis, Sertoli cells are transformed into columnar cells and secrete a spermatophore capsule surrounding the sperm. Spermatophore capsule (C), Sertoli cell nuclei (SEn), spermatids (ST), sperm (SP). Bar = 10 μ m

Fig. 16a-c.

Lobule maturation in embiotocid testes. [a] Early maturation in *Cymatogaster aggregata*, spermatogonia reside at the distal termini of the lobules; spermatocytes and spermatids are more proximal, while sperm are located closest to the efferent ducts. Spermatocysts stretch across the lobule width, and there is no lumen. Bar = 50 μ m. [b] Later in development, in the testis of *Embiotoca jacksoni*, spermatogonia are no longer observed at the distal termini of lobules, spermatocysts with developing sperm in different stages of maturation are randomly observed along the lobule wall, and sperm, organized in spermatozeugmata, within a lumen that has developed after spermiation begins. Bar = 50 μ m. [c] In late maturation of the testis in *Phanerodon furcatus*, the lobules are devoid of developing sperm. All that exists of germ cells are scattered spermatozeugmata within spermatocysts or within the lobule lumina. Occasional spermatogonia are among columnar Sertoli cells. Bar = 50 μ m.



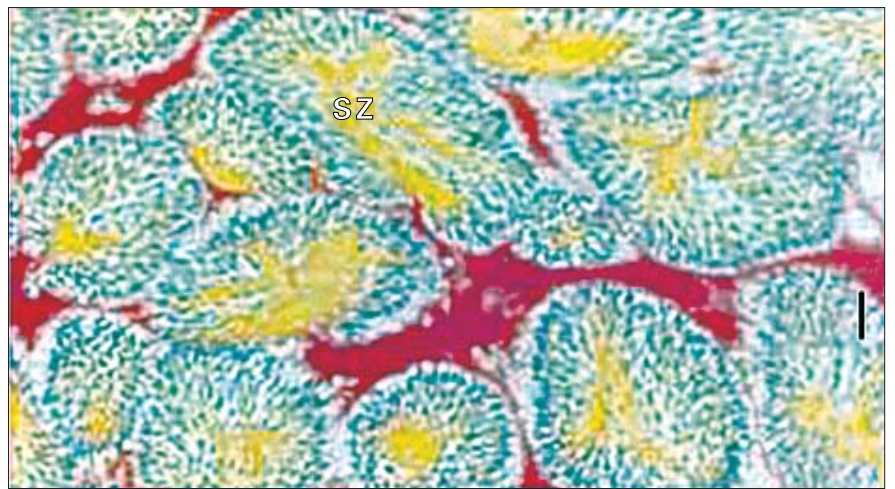
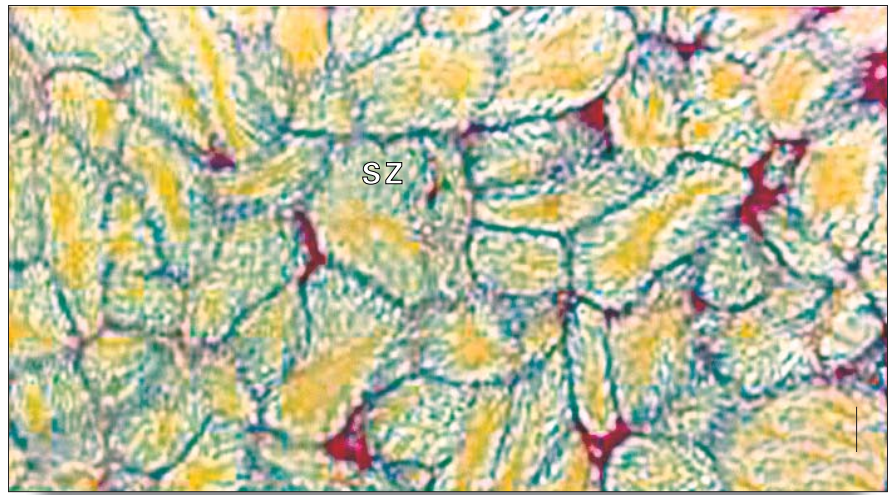
atherinomorph characteristics are clear. The process of testis maturation is not well documented here, indicating that further work needs to be done with multiple species to detail the process.

Armed with a new definition –epithelioid– to describe the distribution of germ and Sertoli cells, the surfperch lobule appears to be an epithelioid assemblage of germ and Sertoli cells early in the maturation process, similar to the Atherinomorpha. As in other neoteleosts, however, (*viz.*, Grier, 1993; Parenti and Grier, 2004), there seems to be a continuous loss of spermatogonia within testis lobules during maturation, leaving them without germ cells, aside from scattered spermatogonia. A discontinuous GE develops as in *Centropomus undecimalis* (Grier, 1993; Grier and Taylor, 1998) and in the striped mullet, *Mugil cephalus* (compare Figs. 1,2). Surfperch Sertoli cells, lining the lobules after germ cells are released as sperm, form a secretory, hypertrophied epithelium –as in the atherinomorphs (Figs. 10,13,15). Hypertrophy of Sertoli cells does not occur in other non-atherinomorph neoteleosts that scatter eggs. This is yet another example in which the function of Sertoli cells becomes modified coinci-

dent with the evolution of internal fertilization and sperm packaging. Within the testis ducts of embiotocids, spermatozeugmata are surrounded periodic acid Schiff-positive secretion (Figs. 16d,e), but this does not penetrate into their centers as it does in the poeciliid, *Cnesterodon decemmaculatus* (Fig. 14).

Ophidiiformes comprise a group of largely marine fishes that have truncated bodies and, in some families, internal fertilization. Spermatozeugmata are formed within the spermatocysts and released into the lobule lumina where they are surrounded by a periodic acid Schiff-positive secretion, as in *Dinematichthys* sp. (Figs. 17a-c) and *Brotula multibarbata* (Figs. 18a-c). The secretion is formed by Sertoli cells (Fig. 18b) and is observed within their cytoplasm when surrounding a spermatozeugma within a spermatocyst. The periodic acid Schiff-positive glycoprotein was misinterpreted as the covering of a spermatophore (Nielsen et al., 1968), but it obviously becomes a secretion surrounding all of the spermatozeugmata within the testis ducts. The periodic acid Schiff-positive secretion is observed in the testis ducts of these internally-fertilizing fishes. It is analogous to the secretions observed in certain atherinomorphs and in surperches. No putative atherinomorph relative has the atherinomorph testis type (see Parenti, 2004), although we consider the survey of testis types among teleost fishes to be preliminary (Parenti and Grier, 2004).

Females: As in males, there are modifications in the female reproductive tract of viviparous fishes. As noted above, both intrafollicular (Poeciliidae) and extrafollicular (Goodeidae, Fig. 10) embryo gestation occurs. In the goodeid *Skiffia bilineata*, there are marked changes in the ovarian GE during gestation (Mendoza, 1943). The epithelial cells become secretory, and a new function associated with viviparity has evolved. These changes in the epithelium are accompanied by an increase in vascularity and swelling in the stroma (ovigerous folds) during gestation. As with other morphological changes associated with viviparity, new function(s) become part of the normal cellular activities within tissues. Cell and tissue types are not created *de novo*. In this instance, the GE remains unchanged relative to its cellular composition and primary function of producing follicles, but the epithelial cells change dramatically during gestation. Here, we reinterpret Mendoza's (1940) research on the gestational cycle in *S. bilineata* (as *Neotoca bilineata*), conclusions that are yet to be corroborated in other



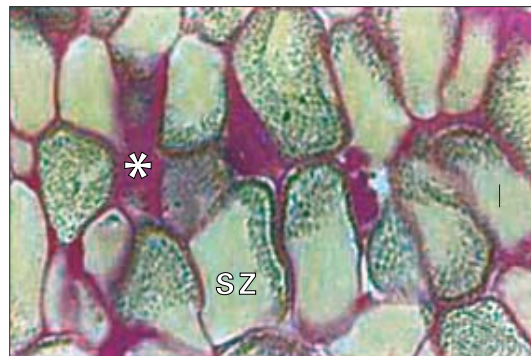
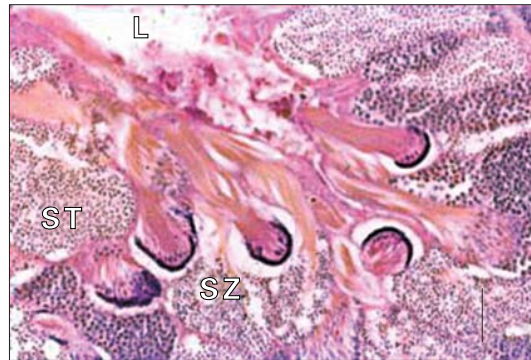
species of viviparous goodeids. The reinterpretation is possible now because of the established role of the GE in producing follicles.

The initial description of a GE in a female fish, in *Centropomus undecimalis* (Grier, 2000), adopted the same criteria that were used to define the male GE and are the criteria that define an epithelium: 1) Epithelial cells are interconnected. As in males, there were observed interdigitated, juxtaposed epithelial cells, tight junctions, and desmosomes. 2) Epithelial cells rest upon a basement membrane. 3) Epithelial cells border a body surface, either the coelom in basal teleosts with gymnovarian ovaries or the ovarian lumen in teleosts with cystovarian ovaries. 4) The GE is avascular in females as in males. Germinal epithelia differ from all other epithelia in that possess both somatic and germ cells. These criteria are repeated here (*vide supra*) to emphasize homology and analogy between male

Fig. 16d-e. Lobule maturation in embiotocid testes. [d] Spermatozeugmata in the testis ducts of *Cymatogaster aggregata* are surrounded by periodic acid Schiff-positive secretion. Bar = 10 μ m. [e] Spermatozeugmata in the testis of *Phanerodon furcatus* are surrounded by periodic acid Schiff-positive secretion. Bar = 10 μ m. Spermatogonia (SG), Spermatocytes (SC), spermatids (ST), sperm (SP), spermatozeugma (SZ)

Fig. 17a-c.

Testis of the ophidiiform, *Dinematichthys* sp. [a] Lobule showing the development of a spermatozeugma within a spermatocyst. Spermatozeugmata occur within the lobule lumen, each surrounded by periodic acid Schiff-positive secretion. Bar = 10 μ m. [b] Several spermatozeugmata are ready to be released into the lobule lumen. Bar = 50 μ m. [c] Spermatozeugmata within the lumen of ducts in the testis are embedded in periodic acid Schiff-positive secretion. Interstitium (I), lobule lumen (L), spermatids (ST), spermatocytes (SC), spermatozeugma (SZA), spermatozeugmata (SZ). Bar = 10 μ m



and female reproductive systems despite their obvious morphological differences. They also underlie the development of the unifying concept (Grier and Lo Nostro, 2000) and the interpretation of a follicle as an epithelial derivative that is always separated from the ovarian stroma by a basement membrane.

In teleosts, the ovarian GE is that epithelium lining the surface of ovarian lamellae or “ovigerous folds,” as they have also been called (Mendoza, 1943). In adult female fish, oogonia are scattered within the GE. Therefore, the ovarian GE is a “discontinuous GE,” a term first defined for establishing male annual reproductive classes in common snook (Taylor et al., 1998; Grier and Taylor, 1998). This distinction between different forms of a GE can be applied

to classify the ovarian GE, but not to establish annual reproductive classes in females as can be done in males. This is owing to the initiation of meiosis and folliculogenesis as a continuous process, at least in female common snook (Grier, 2000), throughout the year. Oocyte stages must be used to establish reproductive classes in female fish. The criteria for establishing annual reproductive classes, based on changes in the male GE, also cannot be applied to atherinomorph males because the testis is an epithelioid arrangement of Sertoli and germ cells.

Through mitosis, oogonia maintain their population within the GE. When entering meiosis and initiating the process of folliculogenesis, oogonia become oocytes and a continual source of new follicles. Each follicle in the fish ovary is composed of an oocyte surrounded by a layer of follicle (granulosa) cells (Grier, 2000), both cell types being derived from the GE. Follicles are separated from the stroma (including the theca which is derived from the stroma) by a basement membrane, as are the cell nests that develop from the GE (Fig. 20b-d). As indicated previously (Grier, 2000; Grier and Lo Nostro, 2000), this definition of a follicle in the fish ovary differs from those that prevail in the fish literature, and is adapted from definitions in numerous histology textbooks. This definition of a follicle precisely reflects its origin from an epithelium, the GE, and reflects homology throughout the vertebrates (Parenti and Grier, 2004).

Ovarian GE occur in ovaries of externally-fertilizing atherinomorphs, the killifishes *Fundulus grandis* (Fundulidae) and *Gnatholebias hoignei* (Rivulidae; Fig. 19a, b; Parenti and Grier, 2004), and the halfbeak *Hemiramphus brasiliensis* (Hemiramphidae; Fig. 19c) and in the ovaries of viviparous atherinomorphs: cuatro ojos, *Anablep anableps* (Fig. 19d), the poeciliids *Poecilia latipinna* (Fig. 19e), *Cnesterodon decemmaculatus* (Fig. 19f), and *Poeciliopsis gracilis* (Uribe et al. 2004), and the goodeid *Ilyodon whitei* (Fig. 20, Uribe et al., 2004). At the level of light microscopy, the ovarian GE in another viviparous fish, *Dinematichthys* sp. (Fig. 19g), is identical to that observed in the atherinomorphs, and GE in these species are morphologically identical to those described in the perciform, *Centropomus undecimalis* by Grier (2000) and the synbranchiform *Synbranchus marmoratus* by Ravaglia and Maggese (2003).

The “emerging constant” of an ovarian GE in fishes (Parenti and Grier, 2004), and an identical process of folliculogenesis, is supported by

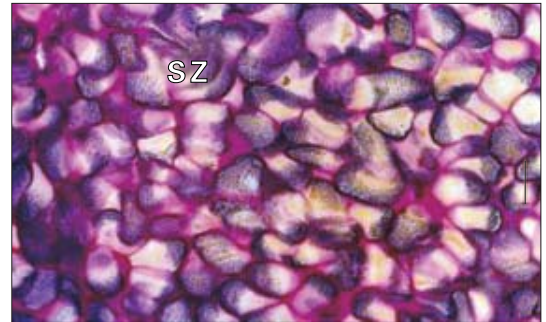
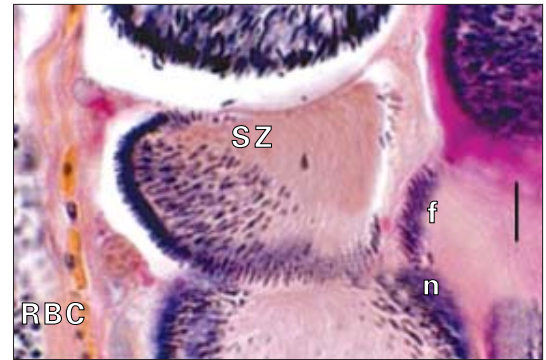
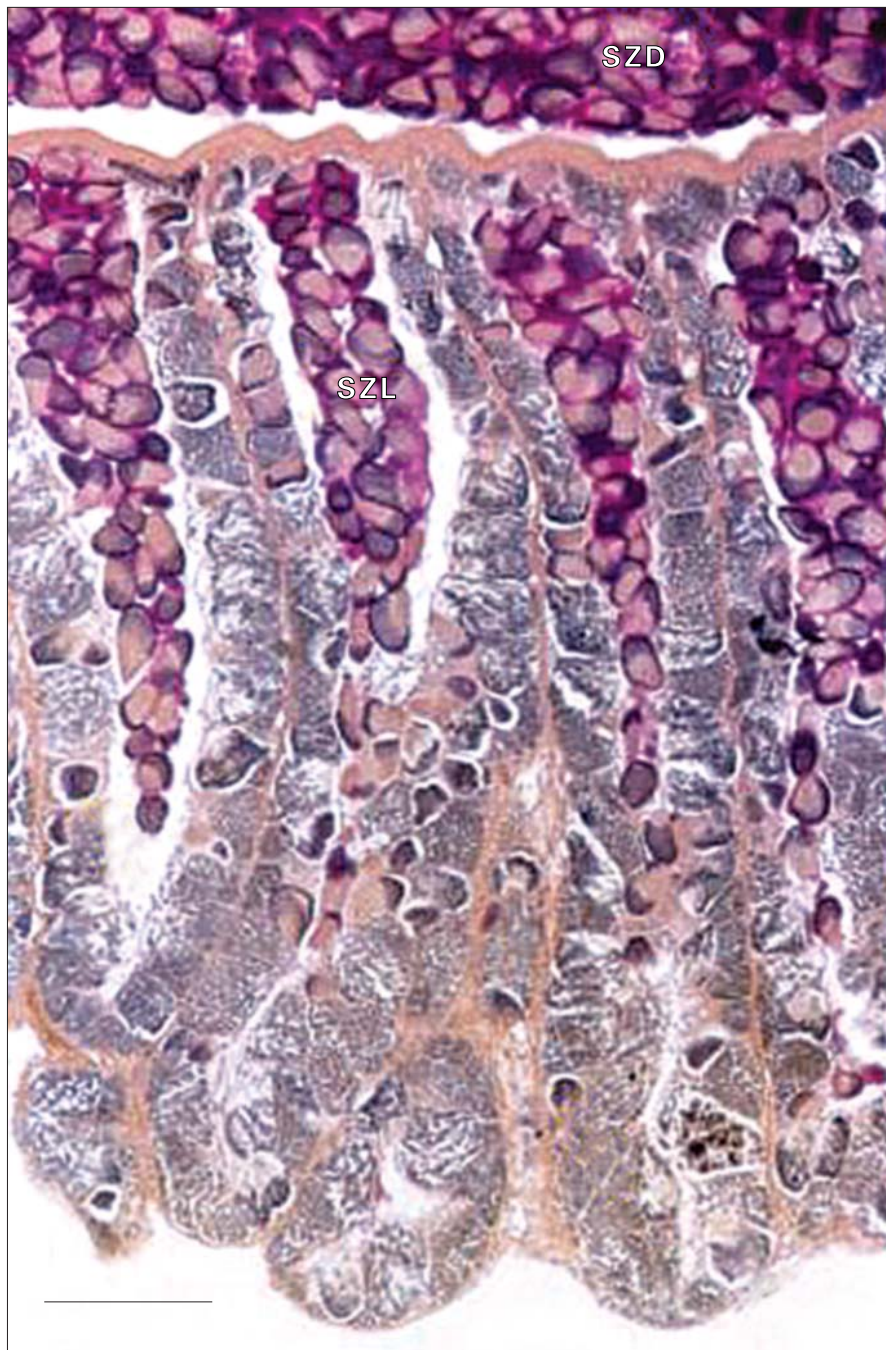


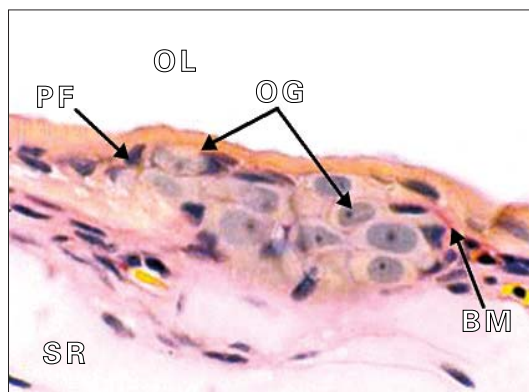
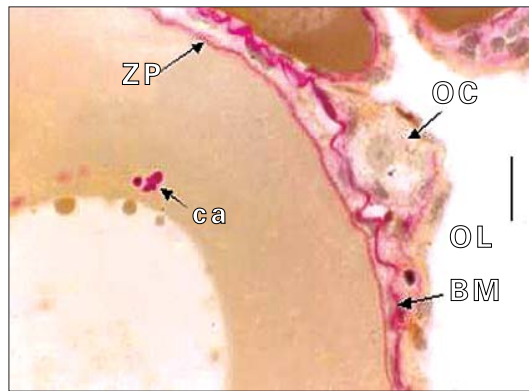
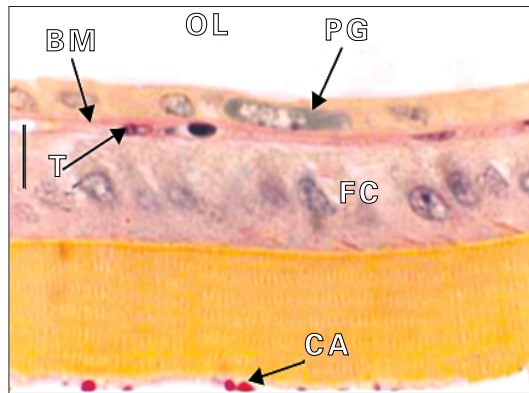
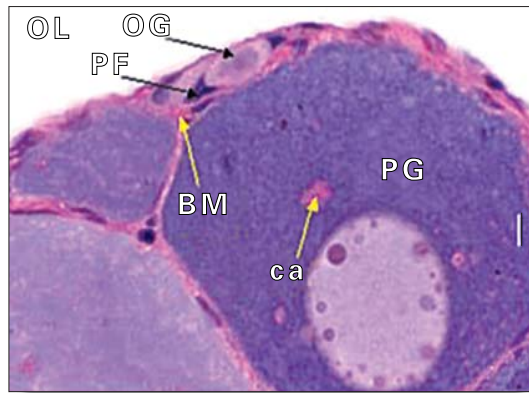
Fig. 18a-c. Early maturation in the ophidiiform, *Brotula multibarbata*. [a] Spermatocysts with different stages of sperm development, extending from the periphery of the testis to the efferent ducts. Spermatozeugmata are observed forming within spermatocysts (yellow arrows), and are within the lobule lumina and the main testis duct. [b] Periphery of a lobule showing periodic acid Schiff-positive secretion in Sertoli cell cytoplasm (thick arrow) and surrounding spermatozeugmata in the lobule lumen (*). Within the interstitium, a capillary with red blood cells is observed. Bar = 10 μ m. [c] Spermatozeugmata within the ducts are surrounded by periodic acid Schiff-positive secretion. Red blood cells (RBC), sperm flagella (f), sperm nuclei (n), spermatogonia (SG), spermatocytes (SC), spermatids (ST), sperm (SP), spermatozeugmata in lobules (SZL) and in ducts (SZA)

the observance of primary growth oocytes in the GE of the cyprinodontiform, *Gnatholebias hoignei* (Fig. 19b) and in the perciform, *Centropomus undecimalis* (Grier, 2000). Indeed, it is self evident that activity of the GE, with regard to mitotic activity of oogonia, transformation of oogonia into oocytes upon the initiation of meiosis, and coincident generation of follicles during reproductive cycles in viviparous fish underlies the sequential production of young and superfetation. The association and

origin of follicles from the epithelium of the ovarian lamellae was reported by Mendoza (1943), describing the germinal tissue in a goodeid, and by Turner (1938) in examining adaptations for viviparity in *Anableps anableps*. In their time and with the limitations of their techniques, the origin of germ cells in the fish ovary became a phenomenon of importance to Mendoza and Turner. The origin of germ cells is likewise now becoming a topic of investigation and appears to be quite similar across taxa.

Fig. 19a-d.

The ovarian GE borders the lumen in six atherinomorphs (first three have external fertilization and the latter three are viviparous) and a viviparous ophiidiiform fish: [a] A single oogonium in the GE in the ovary of *Fundulus grandis* is flanked by prefollicle cells. The GE basement membrane is barely visible. Beneath the GE, a primary growth oocyte, with basophilic cytoplasm and forming cortical alveoli, is observed. Bar = 10 μm . [b] A primary growth oocyte is observed within the GE of the rivulid, *Gnatholebias hoignei* (USNM 245947). The GE rests on a basement membrane that borders thecal cells surrounding a follicle. Columnar follicle cells and a well-developed zona pellucida are observed along with a few cortical alveoli at the oocyte surface. Bar = 10 μm . [c] The GE in the hemiramphid, *Hemiramphus brasiliensis* contains an early diplotene oocyte, and is subtended by a basement membrane. A primary growth oocyte, with forming cortical alveoli, is observed beneath the GE. [d] A single oogonium, flanked by prefollicle cells, resides within the GE of the anablepid, *Anableps anableps*, as well as a cell nest containing oogonia.



The pioneering observations of Mendoza (1943) on the germinal tissue in the goodeid *S. bilineata*, have been corroborated in *Ilyodon whitei* in an extraordinary series of micrographs. The process of folliculogenesis in *I. whitei* is a coordinated progression in the development of a follicle (Figs. 20a-20d), identical to that described in common snook (Grier, 2000). Oogonia reside within the GE (Fig. 20a) and divide mitotically to produce cell nests that remain attached to the GE via a thin cord of cells (Fig. 20a-d). At some point, oogonia enter meiosis, becoming oocytes. A cell nest is composed of either a cluster of oogonia or of early diplotene oocytes and prefollicle cells (derived from the epithelial cells in the GE). Subsequently, primary oocyte growth begins within cell nests (Fig. 20b). Upon initiation of primary growth, an oocyte and its associated prefollicle cells begin to be segregated from the cell nest. Separation is accomplished by the synthesis of an intervening basement membrane (Fig. 20b) between the primary growth oocyte and the remainder of the cell nest. Eventually, the basement membrane will completely encompass the oocyte, forming a follicle that remains attached to the GE (Fig. 20b, c, d). The process by which folliculogenesis begins from a cell nest, and the follicle is later observed to be attached to the GE, is unknown. Prefollicle cells that are associated with oocytes will become follicle cells when folliculogenesis is completed. Mendoza (1943) noted the attachment of “full grown oocytes to the ovarian dells,” an invagination of the GE, seen in Fig. 20c as an infolding of the GE basement membrane.

In *Ilyodon whitei* (Fig. 20a-f), cell nests protrude from the GE towards the stroma but are separated from it by an encompassing extension of the basement membrane of the GE, i.e., a cell nest is encompassed completely by the GE basement membrane as particularly well depicted in Fig. 20b, d. Cells within a cell nest are all derived from and form a continuum with those in the GE. They are not separated from the GE by a basement membrane. In examining dozens of cell nests and follicles in *I. whitei*, a constant morphological feature is observed: the basement membrane that subtends and supports the GE always separates cell nests and follicles, both epithelial derivatives, from the ovarian stroma, a mesodermal derivative. The process of folliculogenesis must involve a continued synthesis of basement membranes to encompass and maintain the separation between cells of different origins.

Here, we demonstrate for the first time that in the fish ovary, follicles maintain an attachment to the GE via their sharing a single basement membrane over a short distance of the follicle surface (Fig. 20b-d). The shared basement membrane both attaches a follicle beneath the GE and separates it from the cells composing the GE. To observe this attachment, appropriate histological sections through a follicle must be observed. The basement membrane of both the follicle and the GE must be cut in cross section, not obliquely, and well stained. This observation of morphology permits an entirely new perspective on follicle development in fish, namely, the continuum between oogonia and process of folliculogenesis beginning within a GE, followed by oocyte maturation within a follicle, ovulation, and formation of the post ovulatory follicle with the follicle and post ovulatory follicle being attached to the GE.

In goodeids, ovulation occurs when a developing embryo is voided into the ovarian lumen from a follicle. The single basement membrane, shared by the follicle and the GE, breaks at the time of ovulation, a separation of the follicle cells and the epithelial cells of the germinal epithelium occurs, and the egg is voided into the ovarian lumen. The follicle cells are left behind. These, and the theca, become a postovulatory follicle (POF) (Fig. 21). Notably, the former follicle cells in a POF continue to be separated from the stroma after ovulation; the POF basement membrane remains intact after ovulation and is continuous with that of the GE (Fig. 21). Furthermore, the epithelial cells of the GE also form a continuum with the former follicle cells of the POF which were derived from the GE. Thus, and until the POF degenerates, the epithelial cells of the GE become reunited with the follicle cells that were once derived from them. Throughout its development, the follicle that is produced by the GE remains sequestered from the stroma, a different tissue layer, by a basement membrane. The separation is maintained even after ovulation. This observation is not dissimilar to that in males where the GE is always separated from the interstitial tissue by a basement membrane.

Far ahead of his time, Mendoza (1943) noted that the flattened cells at the bottom of a “delle” offer “an attenuated and weakened place in the ovigerous fold epithelium through which the egg escapes into the ovarian cavity.” With additional knowledge, we identify that “weakened place” as the location of a single basement membrane

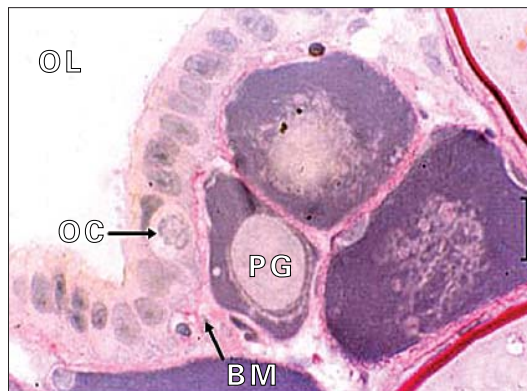
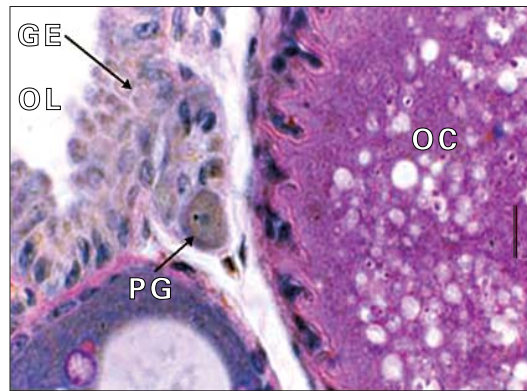
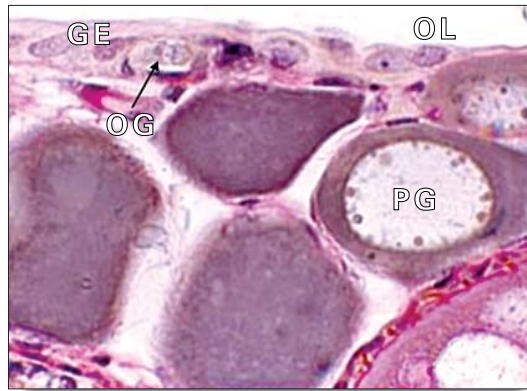
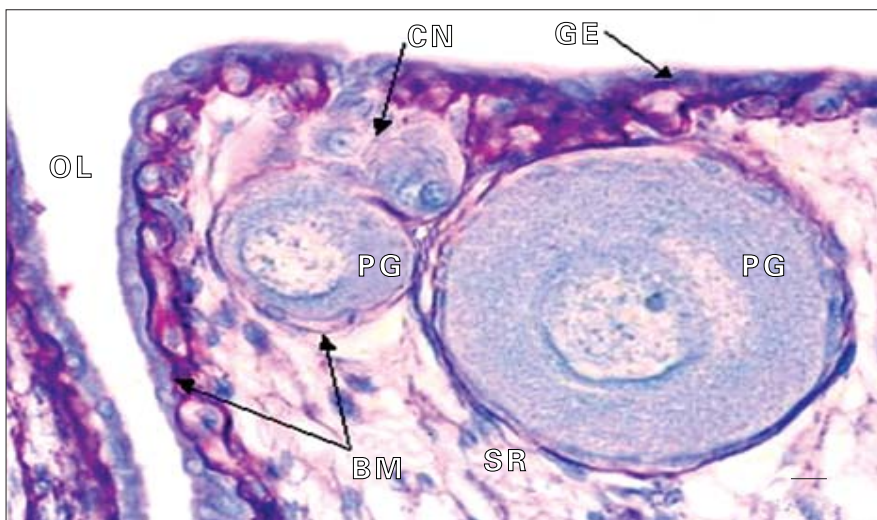
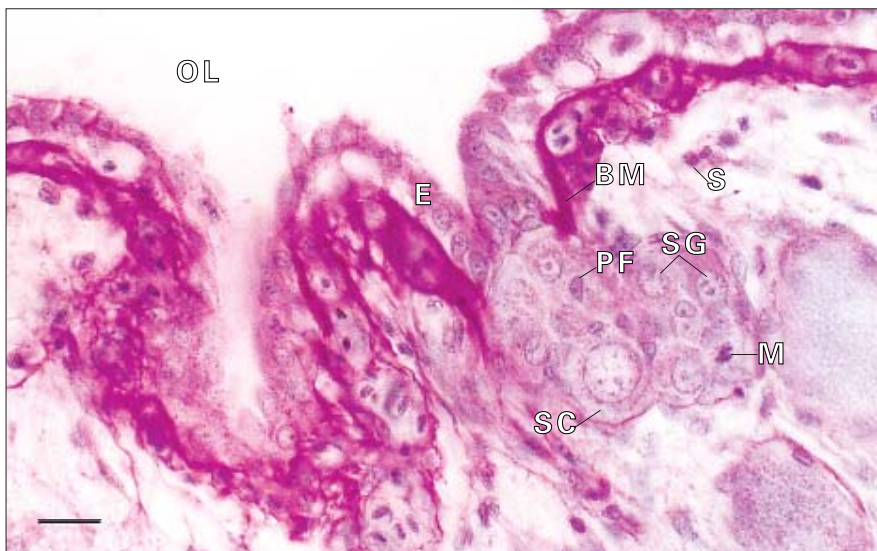
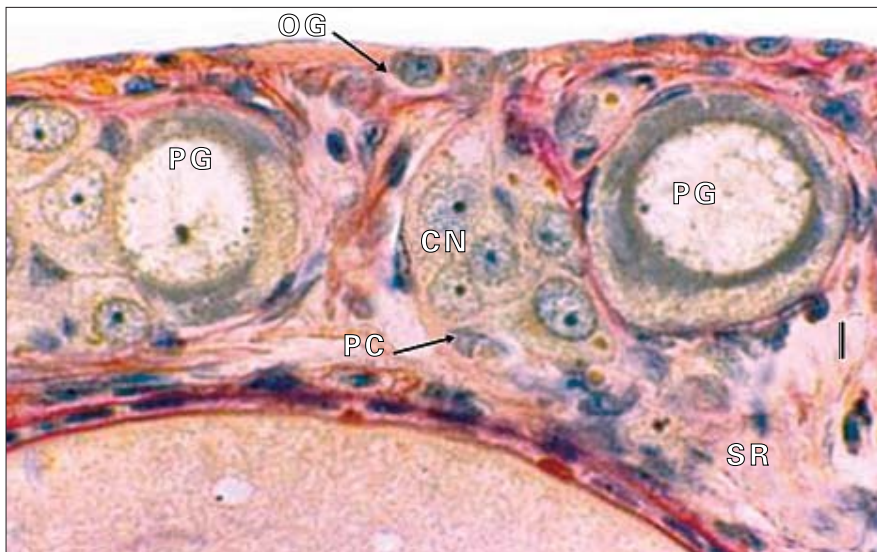


Fig. 19e-g. [e] An oogonium is observed within the GE of the poeciliid, *Poecilia latipinna*, and primary growth oocytes reside beneath the GE. [f] In the poeciliid, *Cnesterodon decemmaculatus*, a prefollicle cells and a single oocyte, just beginning primary growth signified by a thin rim of basophilic cytoplasm around the nucleus, protrudes into the stroma between two follicles with oocytes in different stages of development. The basement membrane is barely visible. Bar = 10 μ m. [g] The GE of the ophiidiiform, *Dinematchthys* sp., is supported by a basement membrane. Within the GE is an early diplotene oocyte, interpreted as such because of the thin rim of basophilic material (RNA) around the inner nuclear membrane. Beneath the GE, primary growth oocytes are observed, two having a well-developed zona pellucida. Bar = 10 μ m. Basement membrane (BM), cortical alveoli (ca), early diplotene oocyte (DO), follicle cells (FC), ovarian lumen (OL), oogonium (OG), primary growth oocyte (PG), prefollicle cells (PF), stroma (SR), thecal cells (T), zona pellucida (ZP)

that is shared between the GE and the follicle. The histological techniques of Mendoza (1943) did not include a stain for basement membranes, and the concept of a GE, including the importance of a basement membrane to separate epithelial derivatives, the follicle, from the stroma, was not arrived at until over a half century later (Grier, 2000). Mendoza (1943) incorrectly surmised that follicle cells were derived from the “subepithelial connective tissue.” Nevertheless, his contribution should be remembered as the origin of follicles emerges as a new area for research in fish and vertebrate reproduction. In



retrospect, the morphology reflects on how an oocyte always ovulates into the ovarian lumen and never into the stroma.

The results of our recent investigations are not limited to the germinal epithelium, but include also oocyte development. In all atherinomorphs surveyed to date, yolk is fluid throughout vitellogenesis, yet another atherinomorph synapomorphy (Parenti and Grier, 2004). In non-atherinomorphs, yolk is granular and becomes fluid only during the cytoplasmic events leading to ovulation.

Conclusions

Morphological similarity, and the uniform nomenclature that can be applied to its description, supports homology. As proposed originally (Grier and Lo Nostro, 2000), the unifying concept stressed unified definitions of morphological structure between taxa. As such, it is recognized that the origin of ovarian follicles from a GE is identical between fish and mammals (Grier, 2000; Parenti and Grier, 2004). The ovarian GE is composed of both somatic cells and germ cells. In fish, the latter may be oogonia and diplotene oocytes that may advance in development as far as the beginning of primary growth. Germ cells and follicle cells in a rabbit were described by Zamboni (1972) as “breaking through” the base-

Fig. 20a-e.

Folliculogenesis in the goodeid, *Ilyodon whitei*: [a] a single oogonium is observed within the GE, and cell nests containing early diplotene oocytes extend from the GE into the stroma; primary growth oocytes are also observed. Bar = 10 μ m. [b] The basement membrane subtending the GE extends around a cell nest, separating it from the stroma. Within the cell nest, one early diplotene oocyte has an enlarged nucleus. [c] A cell nest is observed extending into the stroma and attached to the GE. The cell nest is surrounded by extension of the basement membrane of the GE. Within the cell nest, basement membrane synthesis (large red arrow) is separating a primary growth oocyte from the remaining early diplotene oocytes. An oocyte within a follicle is observed next to the cell nest, connected to the GE, but a single basement membrane (small red arrow) is resolved between the GE and the follicle cells. Bar = 10 μ m. [d] An oocyte and cell nest: the basement membranes are well resolved. The follicle has a periodic acid Schiff-positive basement membrane separating the cells in the GE from the follicle cells. Bar = 10 μ m. [e] Magnified section from the previous micrograph showing prefollicle cells and early diplotene oocytes within a cell nest. These form a continuum with the cells in the GE. Next to the cell nest, the GE basement membrane extends to the surface of the follicle. A single basement membrane is resolved between the follicle cells and the cells of the GE (yellow arrow). Bar = 10 μ m. Basement membrane (BM), cell nest (CN), follicle cells (FC), early diplotene oocytes (DO), oocytes (OC), oogonium (OG), ovarian lumen (OL), prefollicle cells (PC), primary growth oocyte (PG), stroma (SR)

ment membrane of the GE to gain access to and reside in the ovarian stroma. In *Centropomus undecimalis* (Grier 2000) and *Ilyodon whitei*, the process is organized as epithelial cells, oogonia, and oocytes partake in a highly coordinated process of folliculogenesis. The origin of follicle (granulosa) cells from the somatic cell in the GE is documented between vertebrates (Tokarz, 1978) and in mammals (Zamboni, 1972). As more data become available for fishes and other vertebrates, the emerging constant of the GE will become recognized as uniting homologous and essential processes (folliculogenesis, oocyte development, and ovulation) into a continuum that essentially remains unchanged throughout vertebrate evolution. Changes in these processes may be understood as specific modes of reproduction in fishes and other vertebrates.

After ovulation in *Ilyodon whitei*, the basement membrane that surrounded the follicle remains attached to the basement membrane subtending and supporting the GE. Even after ovulation, the follicle cell component of POF remains separated from the stromal tissue by a basement membrane. This new observation stresses the separation between germ layers: a basement membrane separates epithelial (the follicle is derived from an epithelium) derivatives from tissue in other body compartments. It has been suggested that the term “follicle complex” be used to describe the follicle and its surrounding basement membrane and theca (Grier and Lo Nostro, 2000). The definition of a follicle, as being composed only of the oocyte and encompassing follicle cells, agrees with the origin of the follicle from the GE, establishes its homology to follicles in other vertebrate taxa, and also agrees with histology text book definitions (Grier, 2000; Grier and Lo Nostro, 2000).

We describe the GE in male and female teleosts using the same definition, giving examples that germinal epithelia in viviparous fish are essentially the same as those in fish that have an egg-scatter-

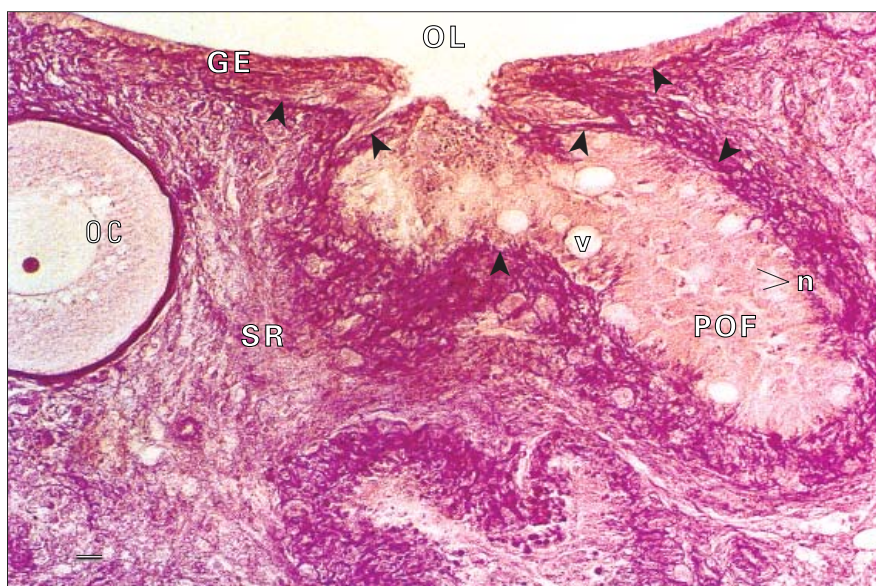


Fig. 21. A post ovulatory follicle (POF) in the ovary of the goodeid, *Ilyodon whitei*: the former follicle cells are vacuolated (v), where lipids presumably dissolved out of the section during histological processing, and cell nuclei (n) are indistinct. There is either extensive synthesis of basement membrane surrounding the POF, or there may also be collagen that stains positively with the periodic acid Schiff reaction. Clearly, a continuous basement membrane underlies the POF and the GE (arrows). The section includes part of a second POF. Stroma (SR), a developing oocyte (OC) surrounded by a periodic acid Schiff-positive zone pellucida. Bar = 10 μ m

ing mode of reproduction, in an array of clades. In all vertebrates, males and females, the GE is composed of somatic cells and germ cells. In males, the somatic cells are the Sertoli cells. In females, they are the epithelial cells that become prefollicle cells associated with a meiotic oocytes or cell nests of oocytes. Finally, prefollicle cells become the follicle cells, surrounding an oocyte, at the completion of folliculogenesis. Of course, the germ cell series begins with gonia, either spermatogonia or oogonia. A major difference between males and females is the existence of a prolonged diplotene stage of development in females. All oocytes are in diplotene of the first meiotic division until just before ovulation when the first meiotic division is completed. Germinal epithelia are the source of gametes in both males and females in spite of morphological diversity among vertebrate gonads, unless an epithelioid association of Sertoli and germ cells exists as in the Atherinomorpha.

Internal fertilization and viviparity arose independently among fish taxa (Parenti, 1981; Lydeard, 1993). Internal fertilization involves the evolution of modified functions of Sertoli cells, which become columnar and secretory, and not the intervention of unusual cell types for the ultimate packaging of sperm. Primarily, Sertoli cell secretions are glycoproteins (periodic acid Schiff –positive), and are seen in numerous taxa that achieved internal fertilization independently of each other.

The distribution of germ cells within the lobular, germinal compartments of male teleosts informs reproductive mode, evolution, and phylogeny. The restricted distribution of spermatogonia within lobules of Atherinomorpha supports their monophyly, but not their phylogenetic relationship to any other taxon with lobular testes (Parenti, 2004; Parenti and Grier, 2004). Similarly, the type of yolk that is formed during vitellogenesis, fluid or globular, is also of significance and may also reflect on monophyly. Morphological diversity, superimposed upon the constancy of fundamental processes (the origin of germ cells from germinal epithelia across taxa and between sexes, for example), reflects underlying genetic diversity.

Morphological diversity, involving conserved evolution of derived functions in cell types present in the ovary, is common in viviparous female fish. In poeciliids, fertilization and the whole of embryonic development is intrafollicular (Turner, 1940), stretching the meaning of ovulation. In goodeids, ovulation

apparently occurs shortly after intrafollicular fertilization, and the embiotocid *Cymatogaster* and the atherinomorphs *Xiphophorus*, *Anableps*, and *Jenynsia*, all have intrafollicular fertilization (Mendoza, 1943). When embryos develop within the ovarian lumen, ovulation must be of developing embryos, not eggs. A cycle in the epithelial cells lining the ovarian lumen (the GE) has been documented by Mendoza (1940) in *Skiffia bilineata*. Hypertrophied cells are involved in producing nourishment for the embryos during the gestational cycle, when the increase in size of individuals may be 1,400 times that of the egg (Wourms, 2004).

We present specific examples of morphological similarity in ovarian germinal epithelia in fish with external fertilization and fish that have evolved a viviparous mode of reproduction independently of each other. The GE is morphologically the same although the ovarian morphology may be quite different across vertebrate taxa. In at least *Skiffia bilineata*, the epithelial cells of the GE change during the gestational cycle (Mendoza, 1940), yet the epithelium remains intact. Morphology changes primarily in the stroma and in eggs. Although the stroma in mammals appears different from that in fish, in both, the stroma forms the theca surrounding follicles (Van Blerkom and Motta, 1979). Morphology has evolved to produce eggs that must develop in a changing environment (evolution of pelagic eggs [eggs that float in saltwater], demersal eggs [attached to a substrate], eggs with attachment filaments, large eggs as in salmonids that are buried in stream beds during a prolonged larval development, eggs surrounded by gelatinous coatings or by calcium in amphibians and reptiles, respectively, and so on. Although they follow different developmental sequences, germinal epithelia in females and males are both derived from somatic cells and germ cells, all derived from a common source. The germ cells are derived from primordial germ cells originating in the hindgut and migrating to the germinal ridges of the developing embryo. Follicle and Sertoli cells are derived from the mesodermal cells in the germinal ridge. Germinal epithelia in males and females are defined the same, except for the epithelioid arrangement observed in male atherinomorphs—an evolutionary modification of the Atherinomorpha.

By application of the periodic acid Schiff staining technique that renders the basement membrane and intercellular connective tissue



bright magenta, a new interpretation of follicle development in the Goodeidae has been established. As a result of the dispersed nature of stromal cells in the goodeid ovary, follicle formation is particularly easy to visualize. Quite unexpectedly, we discovered that follicles remain attached to the underside of the GE throughout their development; each follicle shares a short length of the basement membrane underlying the GE. This is the point where ovulation occurs, at the base of the “delle” (Mendoza, 1943) where there is but a single basement membrane. During ovulation, the shared basement membrane breaks, the follicle enters the ovarian lumen, leaving behind a post ovulatory follicle. The basement membrane, originally surrounding the follicle, remains continuous with that of the GE. During ovulation, a new continuum is formed by the follicle cells that were originally derived from the epithelial cells of the GE, and the epithelial cells composing the GE. Two epithelial cell layers become one until the post ovulatory follicle degenerates. During oocyte development, follicle cells rest upon the basement membrane separation them from the theca, are attached laterally, are avascular, but do not border a lumen as the follicle is filled with the developing oocyte. They should be considered an epithelioid layer of cells.

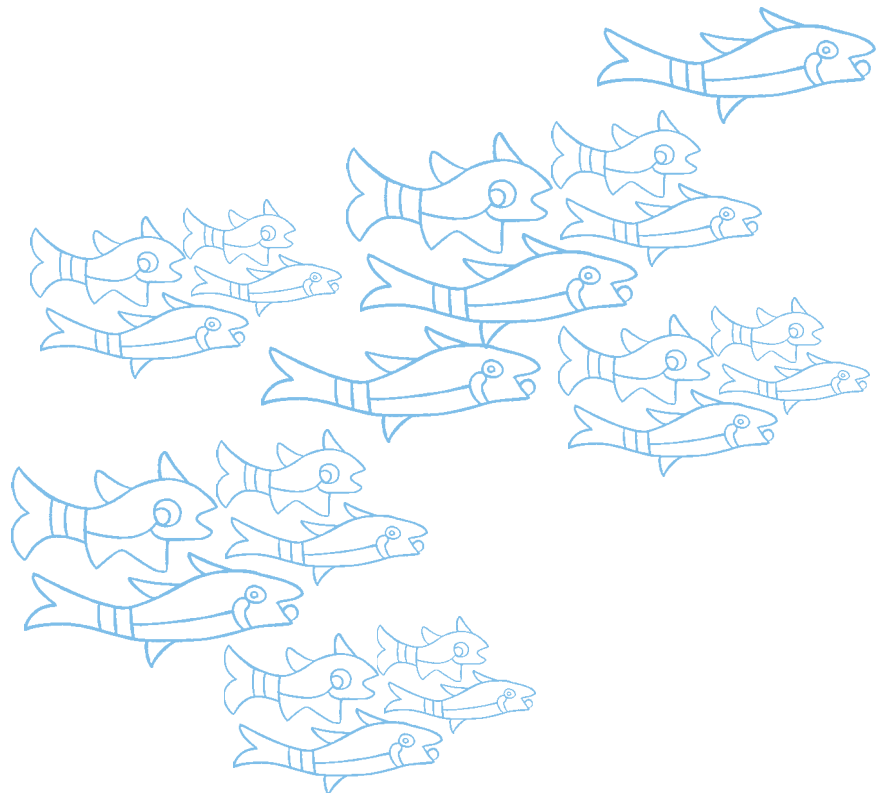
The significance of the new observations is that the epithelial derivative, the follicle (the oocyte and surrounding layer of follicle cells) is always separated from the stroma (a mesodermally derived tissue) by a basement membrane that is derived from that subtending the GE. Folliculogenesis, oocyte development, including the formation of yolk, and the processes leading to ovulation, are continuous. Textbook definitions of a follicle (Grier, 2000) precisely reflect its origin from an epithelium. The follicle extends into but never truly becomes part of the theca, always being separated from it by a basement membrane.

The “unifying concept” is herein expanded to include the origin of gametes in both sexes from a GE and the homology of follicles of viviparous fish, externally fertilizing fish, and all other vertebrates. Mitosis and the initiation of meiosis within a GE underscores fecundity and, most likely, modes of reproduction in fish. Activity of the GE, as it relates to reproductive modes in fish is a new, unexplored area in fish reproduction, particularly in viviparous fish where viviparity is superimposed upon folliculogenesis and oocyte development.

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Voucher specimens have been or will be deposited in the collections of the National Museum of Natural History, Smithsonian Institution (USNM). Some catalogued specimens are referred to in the text with a USNM number; others are cited in Parenti and Grier (2004:table 1). Jeffrey Clayton (USNM) provided valuable technical assistance.



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