Oocyte Development and Staging in the Florida Bass, *Micropterus floridanus* (LeSueur, 1822), with Comments on the Evolution of Pelagic and Demersal Eggs in Bony Fishes

Harry J. Grier[1,2], Wesley F. Porak[3], Jessica Carroll[1], and Lynne R. Parenti[2]

Oocyte development and staging is described from histological preparation of the ovaries of field collected specimens of the Florida Bass, *Micropterus floridanus*. Oocyte development in Florida Bass progresses through six stages beginning with oogonial proliferation and ending with ovulation, the release of eggs. The stages are based on mitosis and meiosis, the core processes of Adaptable Oocyte Staging. After ovulation, the postovulatory follicle complex produces a mucus-like fluid with unknown function; we speculate that it protects the eggs immediately upon ovulation and upon their release into freshwater during spawning. Atresia is rare in the ovaries of reproductive Florida Bass. The release of cortical alveoli after fertilization follows a unique, extended time-release sequence. Oocyte development in the freshwater perciform Florida Bass follows precisely a pattern that has been documented for marine perciform fishes that produce positively buoyant, pelagic, non-adhesive eggs. The eggs of Florida Bass are morphologically like these pelagic eggs in that they have a single large oil globule and no attachment filaments. Yet Florida Bass eggs have small, clear yolk globules and an adhesive egg envelope and are functionally demersal. Thus, classification of an egg as pelagic or demersal should consider its morphology as well as its function, especially if egg type is to be used to interpret phylogenetic relationships or evolutionary patterns. The clearing of yolk due to the uptake of water during the development of actinopterygian eggs, as they progress from a demersal to a potentially pelagic phase, may be a general mechanism of oocyte maturation in teleost fishes.

THE Florida Bass, *Micropterus floridanus* (LeSueur, 1822), is the premier freshwater sport fish in the state. This species is also important ecologically as an apex predator throughout most of Florida's lakes and rivers. The Florida Bass is one of four species of black bass in the family Centrarchidae that together generate approximately 14 million days of angling and $1.25 billion in economic impact annually across the state (USDI FWS, 2006a, 2006b).

Micropterus floridanus is endemic to Florida; the three other native bass species, *M. cataractae* Williams and Burgess, 1999, *M. notius* Bailey and Hubbs, 1949, and the undescribed Choctaw Bass, live in Florida as well as neighboring states. *Micropterus floridanus* is hypothesized to be the sister species of the Largemouth Bass, *M. salmoides* (Lacepède, 1802) in recent phylogenetic analyses (Kassler et al., 2002; Near et al., 2003). Bailey and Hubbs (1949) classified these last two taxa as subspecies of *M. salmoides*: *M. s. salmoides* and *M. s. floridanus*. Mitochondrial sequence divergence between these allopatric taxa was estimated at 3.89%, more than three times that between the Smallmouth Bass, *M. dolomieu* Lacepède, 1802, and the Spotted Bass, *M. punctulatus* (Rafinesque, 1819) (Kassler et al., 2002). This molecular sequence divergence and other characters were used by Barthel et al. (2015) to recognize the Florida Bass and Largemouth Bass as distinct species. The morphological characters that separate the two taxa include differences in number of vertebrae and pyloric caeca (Chew, 1975). Yet taxonomy of the species of bass in Florida remains controversial: the Committee on Names of Fishes (Page et al., 2013), a joint committee of the American Fisheries Society and the American Society of Ichthyologists and Herpetologists, considers *M. floridanus* to be a junior synonym of *M. salmoides*. We follow Kassler et al. (2002), Near et al. (2003), Yokogawa et al. (2005), and Barthel et al. (2015) and treat *M. floridanus* as a valid species to recognize the unique freshwater fish biota of Florida and to encourage its conservation.

Florida Bass are oviparous and are able to mature and spawn within the first year of life (Clugston, 1966; Crumpton et al., 1977), but, typically, they mature at two years or older (Chew, 1974). Peak nesting activity occurs between January and April in peninsular Florida when water temperatures are 18° to 21°C (Clugston, 1966; Chew, 1974). The initiation and duration of reproductive activity is correlated strongly with latitude; reproduction starts earlier and lasts longer in south Florida than in more northern latitudes (Rogers and Allen, 2009). Nests are circular to oval, constructed over firm substrate, and located in water less than 2 m deep, but Florida Bass may broadcast their demersal eggs over submerged aquatic plants, rhizomes of macrophytes, and woody debris (Carr, 1942; Clugston, 1966; Chew, 1974; Bruno et al., 1990). The female leaves the nest after spawning, while the male remains to provide parental care by fanning the eggs day and night. Florida Bass are asynchronous spawners (Parenti et al., 2015). Multiple spawns per individual have been observed in hatchery raceways (M. Matthews, pers. comm., 2017) and in wild populations (Shaw, 2014).

Here we describe oocyte development and staging from histological preparation of the ovaries of field collected specimens of the Florida Bass, *Micropterus floridanus*. The universality of the cell division processes of mitosis and meiosis has recently been incorporated into an Adaptable Oocyte Staging terminology by Grier et al. (2009; Table 1). Cell division is the basis for recognition of six stages of oocyte development: (1) Oogonia Proliferate Stage [mitosis], (2) Chromatin Nucleolus Stage [active meiosis I], (3) Primary Growth Stage [previtellogenesis] and (4) Secondary Growth

[1] Florida Fish and Wildlife Research Institute, 100 8th Avenue SE, St. Petersburg, Florida 33701-5095; Email: (HJG) harry.grier@myfwc.com; and (JC) jessica.carroll@myfwc.com. Send reprint requests to HJG.

[2] Division of Fishes, Department of Vertebrate Zoology, National Museum of Natural History, MRC 159, Smithsonian Institution, P.O. Box 37012, Washington, D.C. 20013-7012; Email: (LRP) parentil@si.edu.

[3] Lakefront Management and Research, 1216 Gray Court, Eustis, Florida 32726; Email: wesporak@aol.com.

Submitted: 25 August 2017. Accepted: 14 March 2018. Associate Editor: M. P. Davis.

 DOI: 10.1643/CG-17-679 Published online: 29 June 2018

Table 1. Stages and steps of adaptable oocyte staging (modified from Grier et al., 2009: table 1). Note that the label for the Late Secondary Growth Step (SG*l*) is italicized to distinguish the letter "l" from the number "1."

Stages	Steps
1. Oogonia Proliferate (OP)	Frequently form cell nests
2. Chromatin Nucleolus (CN)	Leptotene (CNl)
	Zygotene (CNz)
	Pachytene (CNp)
	Early Diplotene (CNed)
3. Primary Growth (PG)	One-nucleolus (PGon)
	Multiple nucleoli (PGmn)
	Perinucleolar (PGpn)
	Circumnucleolar oil droplets (PGod)
	Cortical alveolar (PGca)
4. Secondary Growth (SG)	Early secondary growth (SGe) or Early yolked oocytes
	Late secondary growth (SG*l*) or Late yolked oocytes
	Full-grown oocyte (SGfg)
5. Oocyte Maturation (OM)	Eccentric germinal vesicle (OMegv)
	Germinal vesicle migration to animal pole (OMgvm)
	Germinal vesicle breakdown (OMgvb)
	Meiosis resumes; 2nd arrest (OMmr)
6. Ovulation (OV)	Oocyte emerges from follicle, becomes an egg

Stage [vitellogenesis] (both stages in arrested meiosis), (5) Oocyte Maturation Stage ending with the resumption of meiosis, and the final (6) Ovulation Stage. When abbreviated, the oocyte development stages are designated by two upper case letters and the stages are subdivided into steps that are designated by lower case letters. For example, Primary Growth Stage, Perinucleolar Oocytes Step, is abbreviated PGpn (Grier et al., 2009; Table 1).

Stages proceed through the initiation of meiosis followed by primary and secondary oocyte growth, maturation, and finally the resumption of meiosis (maturation) followed by ovulation. The unique aspect of the new staging scheme is the division of the stages of oocyte development into steps. The stages remain stable because their steps may be modified to adapt the staging to the reproductive diversity that characterizes different fish taxa. The staging was developed to describe oocyte development in marine perciform (*sensu* Page et al., 2013) fishes, such as *Sciaenops ocellatus*, the Red Drum (Grier, 2012). Because the staging is adaptable (Grier, 2012), it has also been used to describe oocyte development in freshwater ostariophysan fishes, the knifefish, *Gymnotus sylvius* (see França et al., 2010), and the catfish, *Pimelodus maculatus* (see Quagio-Grassiotto et al., 2011; Wildner et al., 2013), by eliminating the Oil Droplets Step of the Primary Growth Stage because oocytes of ostariophysan fishes lack oil droplets.

Current knowledge of the reproductive biology of Florida Bass and other species of *Micropterus*, although detailed, does not include description of the process of oocyte development using this scheme. For example, Porter (1997) reported the six stages of final oocyte maturation in Largemouth Bass from Oklahoma but did not describe steps as this protocol had not yet been published, induced spawning using hormones, and did not illustrate histology.

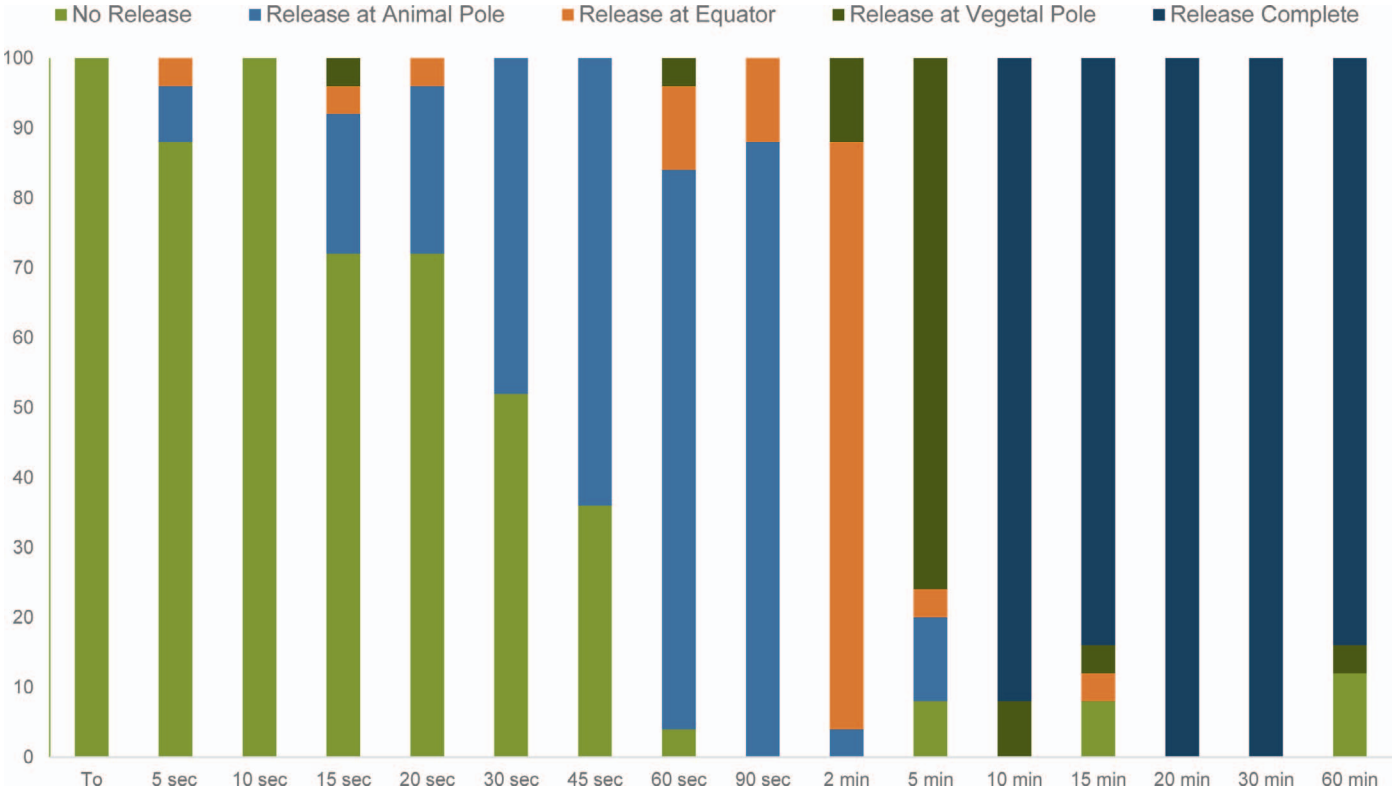
Actinopterygian eggs are often classified using solely functional characters: a pelagic egg floats and a demersal egg sinks (Berois et al., 2011). Morphologically pelagic eggs are typically relatively small, lack filaments, have a thin, smooth egg envelope, clear yolk, and a single oil globule (Breder and Rosen, 1966; Ahlstrom and Moser, 1976). Eggs are broadcast. In contrast, demersal eggs are larger, have a relatively thick egg envelope, and are buried or laid on a substrate to which they may attach by filaments. They may have multiple oil globules. Florida Bass produce eggs that have an adhesive, sticky envelope and that are functionally demersal in contrast to the positively buoyant, pelagic eggs typical of marine percomorphs (Fulton, 1898; Ahlstrom and Moser, 1980; Fyhn et al., 1999; Finn et al., 2000, 2009; Grier, 2000, 2012). Yet the eggs of Florida Bass are morphologically like pelagic eggs of marine teleosts in that they have a single large oil globule and no attachment filaments. Therefore, Florida Bass were collected to document oocyte development to further understand how this process leads to the production of a morphologically pelagic, yet functionally demersal egg, and to address the significance of these egg types in fish phylogeny and evolution.

MATERIALS AND METHODS

Fish collection.—Quarterly collections of Florida Bass were made in 2014–2015 from water bodies within the Ocklawaha Chain of Lakes, including lakes Dora, Eustis, Beauclair, Apopka, and Griffin in Lake County, central Florida. A minimum of three males and three females were collected at each event in February to early March (during the spawn), May to June, September to October, and December to early January. Adult males ranged between 318 mm and 419 mm TL (total length); adult females ranged between 483 mm and 597 mm TL. Florida Bass were collected using a boat-mounted electroshocker. After individuals were landed in the fishing boat, specimens to be used for gonad histology were anesthetized in a solution of MS-222 (Tricaine-S) at a minimum concentration of 330 mg/l and sacrificed by pithing. Fish were collected in accordance with the Guidelines for the Use of Fishes in Research of the American Fisheries Society and the Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research of the American Society of Ichthyologists and Herpetologists.

Fixation and histology of gonads.—The gonads were removed and fixed in Trump's glutaraldehyde and formaldehyde (McDowell and Trump, 1976). Ovaries were sliced into smaller pieces to facilitate faster fixative penetration. Fixation was for, minimally, 24 h after which gonads were cut to an appropriate size for embedding, dehydrated in a series of graded alcohols to 95%, and then embedded in the plastic resin JB-4 (Polysciences, USA) glycol methacrylate. The embedded tissue was sectioned at 5 μm thickness and stained with hematoxylin and eosin (H&E), metanil yellow-periodic acid Schiff-hematoxylin (PAS/MY; Quintero-Hunter et al., 1991), or the reticulin stain (RET; Puchtler and Waldrop, 1978; Vidal, 1988) that clearly distinguishes tissue compartments, an epithelium and underlying tissues, as illustrated previously for gonad development (Mazzoni et al., 2014). Histological sections were examined with an Olympus BX53 microscope equipped with a DP-72 digital camera and using Olympus cellSens version 1.3 imaging software.

Table 2. *Micropterus floridanus*: time course of cortical alveoli release. The time release of cortical alveoli at the animal pole, the egg equator, and the vegetal pole is depicted between 5 seconds through 60 minutes. Actual fertilization of an egg does not correspond instantaneously to the time intervals, but those eggs with no release after five minutes may be considered infertile. Release of cortical alveoli begins at the animal pole, then to the egg equator, and finally to the vegetal pole after ten minutes.



Fertilization.—Fish were collected during the spawning season in March 2014, at Starke Lake, Orange County, Florida, to study fertilization. The abdomens of adult females were squeezed gently to determine if eggs would flow easily from the ovary, indicating that ovulation had taken place. One female approximately 56 cm TL was strip-spawned. Infertile eggs were fixed as they were stripped from the female. Other eggs were stripped into a bowl with freshwater. A single testis was dissected from a male and placed on ice. The testes were dissected into pieces in a separate bowl, and water was added to activate the sperm. The bowl was swirled in a circular motion and, as the contents of the bowl were broadcast over the eggs, a timer was started. Eggs were fixed at 5, 10, 15, 20, 30, 45, 60, and 90 seconds and again at 2, 5, 10, 15, 20, 30, and 60 minutes to study cortical alveoli release and changes in egg morphology. The fixed eggs were processed histologically as above. Numerous eggs were embedded in a single block at each time interval and sectioned *en masse*. Although arranged randomly, only sections from animal to vegetal pole were evaluated for Table 2.

Terminology.—The outer membrane of the fish egg is called alternatively the chorion, zona pellucida, or zona radiata. Because the term "chorion" is used also for a vertebrate embryonic membrane, we reject it here. We prefer the term zona pellucida prior to ovulation, and egg envelope after ovulation, for this tough outer egg membrane in fishes. Breder and Rosen (1966) incorrectly identified the eggs of viviparous taxa as "non-chorionated." The zona pellucida of viviparous taxa is thinner than that of oviparous taxa, but it is present (e.g., Parenti et al., 2010).

Disposition of specimens.—A set of histological slides and formalin-fixed voucher specimens will be deposited in the Division of Fishes, National Museum of Natural History, Smithsonian Institution, Washington, D.C.

RESULTS

The ovary.—The Florida Bass ovary, as observed in a September-caught, regressed female (Fig. 1A), is composed primarily of circumferential ovarian lamellae, each of which has a core composed of an extension from the ovarian wall, appearing to be smooth muscle. Lateral to the core, each lamella has numerous primary growth oocytes and a few large vacuoles that are probably remnants of developed, now atretic, follicles that did not ovulate during the previous spawning season. The ovary is suspended from the dorsal body wall by a mesentery, the mesovarium. The latest stage and step of oocyte development present in September is the Primary Growth Stage, Cortical Alveolar Step (PGca); oocytes are just beginning to form periodic acid Schiff (PAS)-positive cortical alveoli (Fig. 1B) near the germinal vesicle. The cortical alveoli are barely resolved in what would otherwise be a perinucleolar oocyte (PGpn), the "Steps" being designated by the latest ooplasmic event.

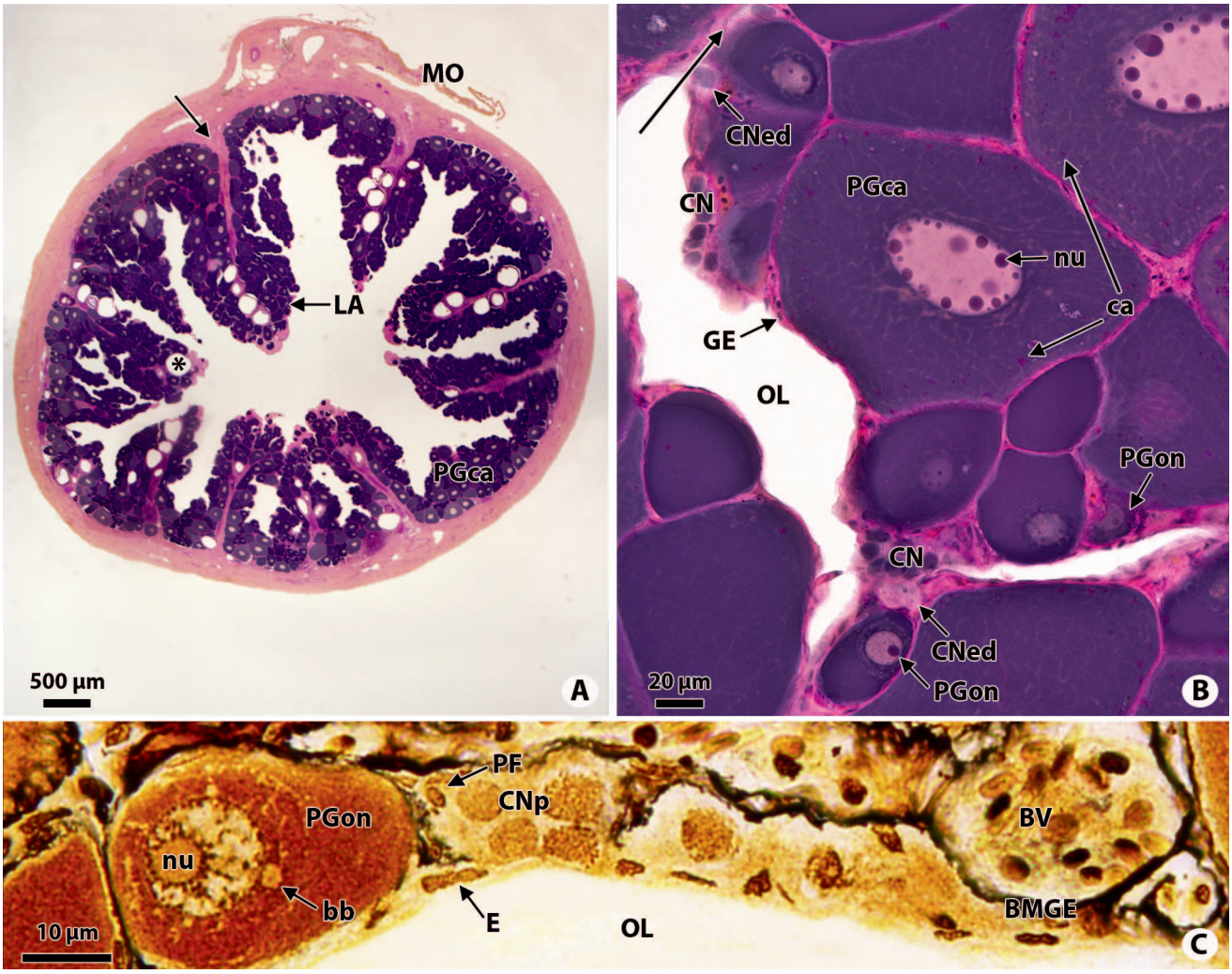


Fig. 1. *Micropterus floridanus*. (A) Ovary transverse section to illustrate basic morphology, here in a regressed female collected in September. The ovary is suspended from the dorsal body wall by the mesovarium. The encompassing ovarian wall has multiple extensions (arrow) into the lamellae. Primary growth oocytes are on either side of these extensions. Spherical, clear spaces (one is marked *) are oocyte atresia remnants from the previous breeding season. PAS/MY, Bar = 500 µm. (B) The principal oocytes in the ovary have perinucleolar nuclei (nu); small PAS-positive "granules" in the ooplasm indicates that they are in primary growth and have developing cortical alveoli. Two primary growth oocytes, each with one nucleolus, are visible. A germinal epithelium (GE) lines the ovarian lamella and borders the ovarian lumen. Within the GE are oocyte cell nests and two chromatin nucleolus, early diplotene oocytes. Long arrow in upper left points to extension of the germinal epithelium into the stroma. PAS/MY, Bar = 20 µm. (C) The edge of an ovarian lamella illustrates pachytene oocytes in the Chromatin Nucleolus Stage of development. A one nucleolus, primary growth oocyte is within the germinal epithelium that is separated from stroma by a black basement membrane and from the ovarian lumen by epithelial cells that become prefollicle cells when associated with oocytes. A blood vessel underlies the germinal epithelium. RET, Bar = 10 µm. bb, Balbiani body; BMGE, germinal epithelium basement membrane; BV, blood vessels; ca, cortical alveoli; CN, cells nest; CNed, Chromatin Nucleolus Stage, early diplotene step oocyte; CNp, Chromatin Nucleolus Stage, pachytene step oocyte; E, epithelial cells; LA, lamella; MO, mesovarium; nu, single nucleolus; OL, ovarian lumen; PF, prefollicle cell; PGca, Primary Growth Stage, cortical alveolar step oocyte; PGon, Primary Growth Stage, one nucleolus step oocyte.

The lamellar epithelium bordering the ovarian lumen is a germinal epithelium. Cell nests with oocytes in the chromatin nucleolus stage of development (beginning meiosis; Fig. 1B) are within this epithelium. In some cases, the meiotic oocytes extend from the germinal epithelium into the lamella. Therefore, not all follicles develop along the surface germinal epithelium. When stained for reticulin (Fig. 1C), a black basement membrane clearly subtends the germinal epithelium, separating it from stroma and blood vessels. The epithelium is composed of epithelial cells. When these associate with germ cells, here pachytene oocytes and a primary growth oocyte, they become prefollicle cells. Stained for reticulin, the primary growth oocyte has a deep, golden-brown ooplasm, a large, less dense ooplasmic body, the Balbiani body, and a single nucleolus that appears to be radiating rRNA (Figs. 1C, 2E).

Oogonia (Fig. 2A) are scattered throughout the germinal epithelium, but are not numerous. They may divide mitotically and then enter meiosis to yield a cell nest of synchronous oocytes in pachytene of the first meiotic division (Fig. 2B). Oogonia may also enter meiosis directly without producing a cell nest, as when single oocytes are observed undergoing folliculogenesis (Fig. 2C, D). After pachytene, oocytes enter the Early Diplotene Step in the Chromatin Nucleolus Stage (CNed, Fig. 2C). Early diplotene oocytes appear similar to single oogonia except that they are larger (compare Fig. 2A and 2C). Each is characterized by a single nucleolus and "clear" cytoplasm or ooplasm, respec-

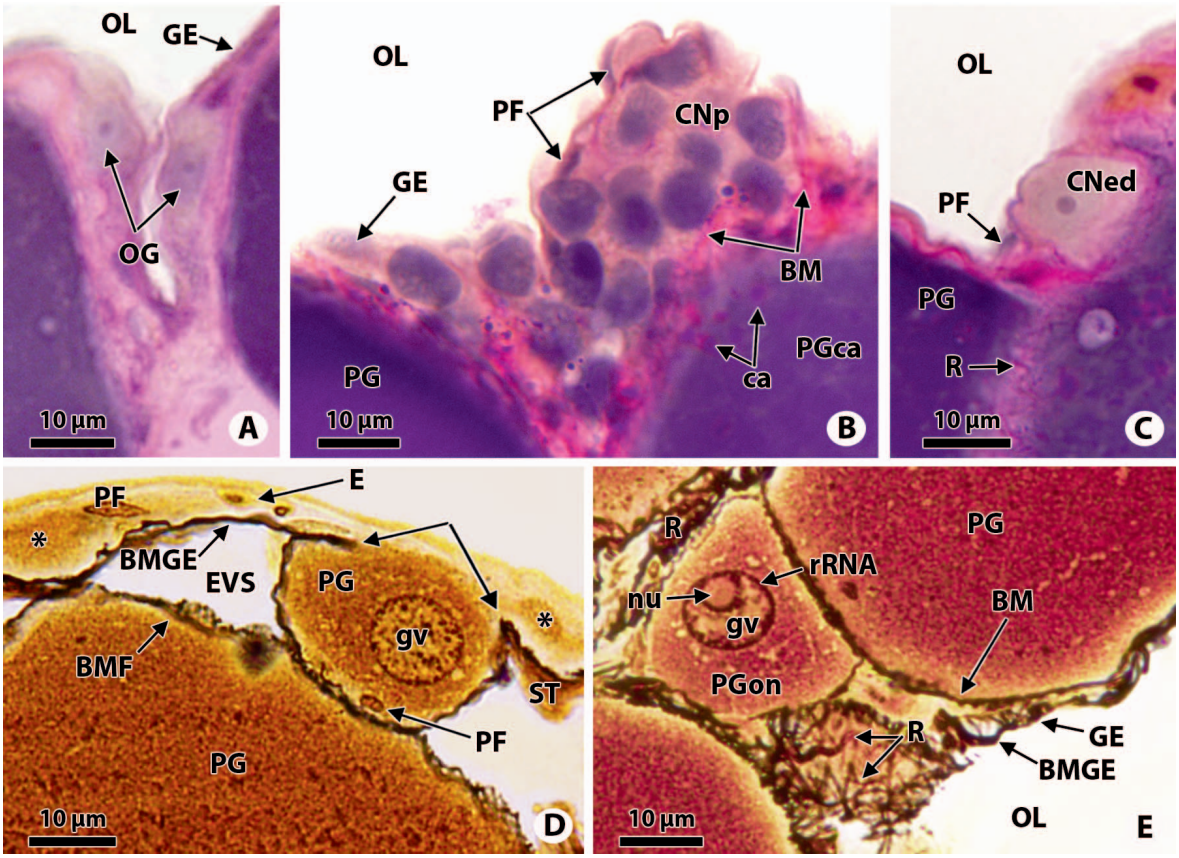


Fig. 2. *Micropterus floridanus*. (A) The germinal epithelium is the border between ovarian lamellae and the ovarian lumen. Two oogonia are observed within the germinal epithelium, and dark-staining cell nuclei on either side are either epithelial cells or prefollicle cells. PAS/MY, Bar = 10 µm. (B) A cell nest containing numerous pachytene oocytes in the Chromatin Nucleolus Stage is separated from the ovarian lumen by prefollicle cells. The basement membrane subtending the cell nest is PAS-positive, but hardly resolved. A primary growth oocyte and an oocyte in the cortical alveolar step of primary growth, with PAS-positive cortical alveoli, are visible. PAS/MY, Bar = 10 µm. (C) After pachytene, oocytes enter the early diplotene step in the Chromatin Nucleolus Stage (CNed). A single CNed oocyte is observed associated with a prefollicle cell. Numerous fibrils, reticular fibers, and two primary growth oocytes with basophilic ooplasm are observed. PAS/MY, Bar = 10 µm. (D) Folliculogenesis is the process by which developing, primary growth oocytes are separated from the germinal epithelium by a basement membrane. The developing follicle maintains contact with the germinal epithelium containing epithelial cells and those that have associated with other primary growth oocytes to become prefollicle cells. The fully-formed, primary growth oocyte in a follicle is surrounded by a basement membrane, and the germinal epithelium is subtended by a basement membrane. Ooplasm of two primary growth oocytes in the germinal epithelium (each *) are sectioned tangentially. When the basement membrane completely encloses the developing follicle (between pair of arrows), a follicle is formed and its prefollicle cells become follicle cells. RET, Bar = 10 µm. (E) A Primary Growth Stage, one nucleolus step oocyte has a spherical germinal vesicle that is rimmed with ribosomal ribonucleic acid (rRNA). The oocyte is surrounded by a basement membrane that appears to be attached to the germinal epithelium by reticular fibers that emanate from the basement membrane of the germinal epithelium. RET, Bar = 10 µm. BM, basement membrane; BMF, follicle basement membrane; BMGE, germinal epithelium basement membrane; ca, cortical alveoli; CNed, Chromatin Nucleolus Stage, early diplotene step oocyte; CNp, Chromatin Nucleolus Stage, pachytene step oocytes; E, epithelial cells of the germinal epithelium; EVS, extravascular space; GE, germinal epithelium; gv, germinal vesicle; nu, nucleolus; OG, oogonia; OL, Ovarian lumen; PF, prefollicle cells; PG, Primary Growth oocyte; PGca, Primary Growth Stage, cortical alveolar step oocytes; PGon, Primary Growth oocyte with one nucleolus; R, reticular fibers; ST, stroma.

tively. They always associate with prefollicle cells which are derived from the germinal epithelial cells. Many of the early diplotene oocytes are single cells not found in a cell nest. They enter primary growth prior to the completion of folliculogenesis (Fig. 2D); when encompassed by a basement membrane, folliculogenesis is completed in the One Nucleolus Step in the Primary Growth Stage. Then, the prefollicle cells are follicle cells, and the follicle is composed of the oocyte and its encompassing follicle cells surrounded by a basement membrane that separates it from stroma, from which thecal cells are derived. Within a follicle, One Nucleolus Step oocytes may have the entire nucleolus and inner nuclear membrane rimmed in black silver deposit when stained for reticulin (Fig. 2E) or deep basophilia when stained with PAS/MY or H&E. This is the ribosomal ribonucleic acid (rRNA) that, when transported to the ooplasm along with other RNAs, renders it, in turn, basophilic, initiating primary oocyte growth. Morphologically, it also appears that follicles may "migrate" away from the germinal epithelium, but reticular fibers from the germinal epithelium and also those within the stroma appear to inter-connect follicles and stromal components (Fig. 2E).

The mechanism by which oocytes become perinucleolar oocytes involves the "fission" of initially large nucleoli into progressively smaller units (Fig. 3A, B). By December, cortical alveolar oocytes, fixed in Trump's solution, possess numerous large cortical alveoli (Fig. 3C) that stain intensely with the PAS/MY technique. Also, oil droplets surround the germinal vesicle, appearing as small, clear vacuoles. December is the month when fully developed, cortical alveolar oocytes are the largest and the latest oocytes in the ovary (Fig. 4). These oocytes have a germinal vesicle with an irregular contour and

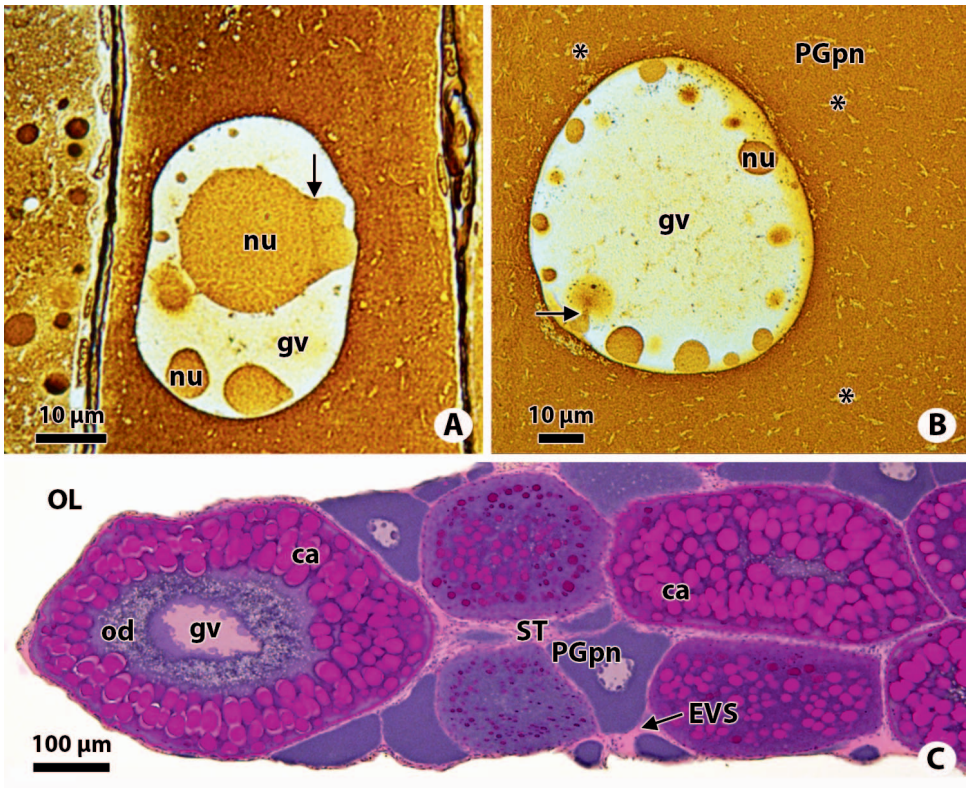


Fig. 3. *Micropterus floridanus*. (A) As primary growth proceeds, nucleoli within the germinal vesicle become numerous; they originate from division (arrow) of the larger nucleoli. RET, Bar= 10 µm. (B) Initially, oocytes in the perinucleolar step of primary growth have a germinal vesicle with a smooth contour. A smaller nucleolus is separating (arrow) from another nucleolus. Clearer areas in the ooplasm (*) are probably due to breakup of the Balbiani body. RET, Bar = 10 µm. (C) By December, full-grown, cortical alveolar oocytes represent a major component in the ovarian lamellae and have large, PAS-positive cortical alveoli. Oil droplets surround the germinal vesicle. Stroma contains numerous cells and the extravascular space. PAS/MY, Bar = 100 µm. ca, cortical alveoli; EVS, extravascular space; gv, germinal vesicle; nu, nucleoli; od, oil droplets; OL, ovarian lumen; PGpn, Primary Growth Stage, perinucleolar step oocyte; ST, stroma.

small nucleoli oriented around the periphery. Numerous oil droplets surround the germinal vesicle. These are surrounded, in turn, by large, more peripheral cortical alveoli. The cortical alveolar oocyte is encompassed by a zona pellucida and a follicle cell layer to form the follicle. In turn, the follicle is encompassed by a basement membrane and a stroma-derived theca. The follicle, basement membrane, and theca form a follicle complex which has a small region in which thecal cells are absent. Here, the basement membrane of the follicle and germinal epithelium join to become one (Fig. 4). Within the follicle complex, the basement membrane surrounding the follicle cells was poorly illustrated in the PAS-stained follicle complexes and appears to be highly "broken," but is stained strongly with RET (Fig. 2D, E).

By January, in addition to previtellogenic oocytes in primary growth, early and late secondary growth oocytes are also present in the Florida Bass ovary. Yolk globules of early secondary growth oocytes are not full-size, whereas some are full-size in late secondary growth oocytes (SG*l*; Fig. 5). There is also a concomitant size difference between early

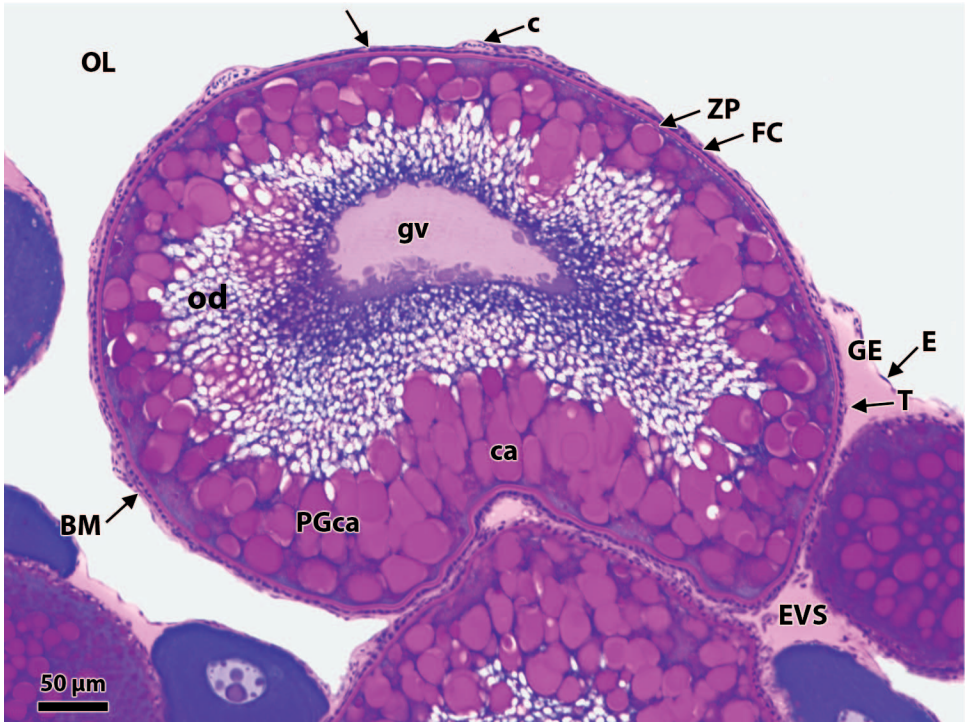


Fig. 4. *Micropterus floridanus*. A cortical alveolar oocyte from a Florida Bass collected in December. Cortical alveoli are peripheral. The germinal vesicle is surrounded by oil droplets. The oocyte is surrounded by a layer of follicle cells, an acellular zona pellucida and a theca, barely resolved at the magnification of the micrograph. Capillaries are within the theca. The arrow points to the site where ovulation will occur: the only cells present are the follicle cells and those of the germinal epithelium. Epithelial cells separate the germinal epithelium from the ovarian lumen. PAS/MY, Bar = 50 µm. BM, basement membrane; c, capillaries; ca, cortical alveoli; E, epithelial cells; EVS, extravascular space; FC, follicle cells; GE, germinal epithelium; gv, germinal vesicle; od, oil droplets; OL, ovarian lumen; PGca, Primary Growth Stage, cortical alveolar step oocyte; T, theca; ZP, zona pellucida.

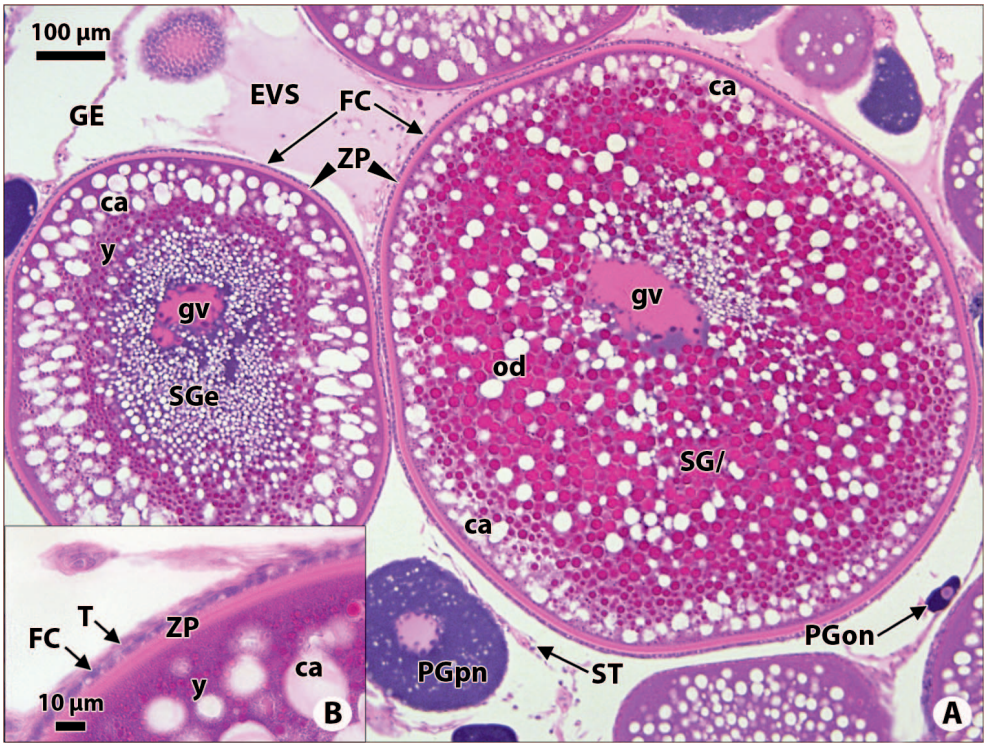


Fig. 5. *Micropterus floridanus*. (A) A single Primary Growth Stage, one nucleolus step oocyte is attached to the germinal epithelium. Within the ovarian lamella is an extensive extravascular space and stromal cells are next to a Primary Growth Stage, perinucleolar step oocyte. An early secondary growth oocyte, with small yolk globules, is next to a late secondary growth oocyte with maximum-sized yolk globules. Oil droplets surround the germinal vesicles which have irregular outlines. With H&E staining, cortical alveoli around the oocyte periphery are primarily clear vesicles, some with eosin staining. H&E, Bar = 100 µm. (B inset) Thecal cells associate with stroma (arrow) from an oocyte with a well-defined zona pellucida and cortical alveoli. H&E, Bar = 10 µm. ca, cortical alveoli; EVS, extravascular space; FC, follicle cells; GE, germinal epithelium; gv, germinal vesicle; od, oil droplets; PGon, Primary Growth Stage, one nucleolus step oocyte; PGpn, Primary Growth Stage, perinucleolar step oocyte; SGe, Secondary Growth Stage oocyte, early step; SG*l*, Secondary Growth Stage oocyte, late step; ST, stromal cells; T, theca; y, yolk globules; ZP, zona pellucida.

and late secondary growth oocytes, each encompassed by a zona pellucida and a follicle cell layer. They have numerous cortical alveoli around their periphery and numerous oil droplets deeper in the ooplasm. Of interest, oil droplets appear as empty vacuoles in H&E-stained tissue (Fig. 5). Most cortical alveoli also appear as identical, empty vacuoles, but some have a light eosinophilic tinge. They are intensely PAS-positive (Figs. 3C, 4). Detailed examination of histology reveals that each follicle is not free within the stroma; one mechanism of attachment appears to be the stromal tissue (Fig. 5B) which binds tissue elements loosely together within the extravascular space (Figs. 5A, 6). Reticular fibers (Fig. 2E) may also have the same function.

The first visible sign of oocyte maturation in a follicle is when oil droplets begin to coalesce to form oil globules (Fig. 7); the germinal vesicle becomes surrounded by oil globules (Fig. 7B). As coalescing continues, a single oil globule begins to emerge (Fig. 7C). Until this point, the appearance of yolk does not change appreciably. Yet, in preovulatory oocytes (Fig. 7D), yolk globules are polymorphic and larger than observed previously and become clear during maturation. There is typically a single oil globule, and cortical alveoli remain peripheral in the preovulatory oocyte.

A comparison between an oocyte in maturation and one that is preovulatory (Fig. 6) illustrates the marked differences between them. The maturing oocyte has oil droplets and coalescing oil droplets, now oil globules. Although the preovulatory oocyte has a single oil globule, there may be a single large oil globule that is accompanied by one or more smaller oil globules (Fig. 8A). Clear yolk globules in the preovulatory oocyte are larger and tend to be more elongate than those in the oocyte that is beginning maturation (Fig. 6). Oocyte investments consist of the zona pellucida, follicle cells, basement membrane, and a theca, comprising a follicle complex; the latter two are difficult to resolve at the magnification of the micrograph. Stromal tissue interconnects these follicles (Fig. 6).

Ovulation.—Ovulation is the process by which an oocyte emerges from the follicle to become an egg or ovum. At the beginning of ovulation (Fig. 8A, B), the follicle cells, basement membrane, and cells of the germinal epithelium "part" to create an opening for the oocyte to emerge into the ovarian lumen. At ovulation, the follicle cell layer and the epithelial cell layer of the germinal epithelium are joined (Fig. 8B). We note that the follicle cells appear vacuolated (Fig. 8B) prior to ovulation because there is a mucus-like, clear secretion within the ovarian lumen that was observed upon dissection but was not fixed in the histology preparation.

After ovulation, the oocyte investments that remain in the ovarian stroma comprise a postovulatory follicle complex (abbreviated POC; Fig. 9); these are remnants of the former follicle complex prior to ovulation. The POC includes the postovulatory follicle (POF), strictly being the former follicle cells. The postovulatory follicle is separated from the postovulatory theca (POT) by a basement membrane. Images of these cell layers from a follicle complex that possesses a

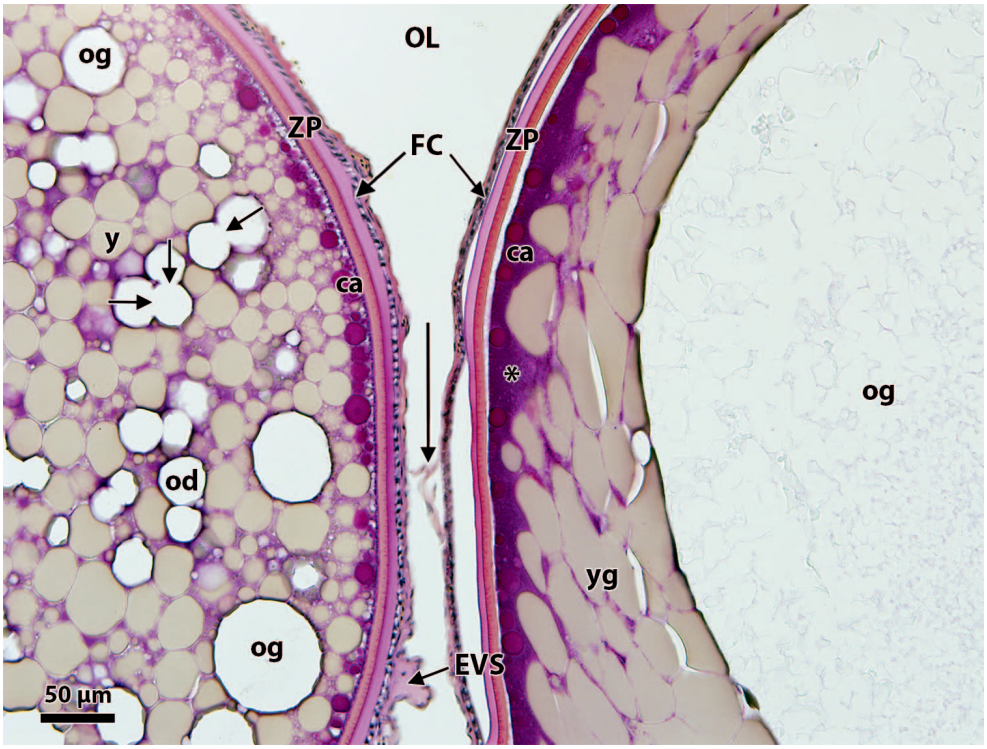


Fig. 6. *Micropterus floridanus*. Portions of juxtaposed oocytes. The oocyte on the left is in maturation, with oil droplets fusing (small arrows) to become larger oil globules; the oocyte on the right is preovulatory, with a single, large oil globule. The oocytes are attached by stromal cells (large arrow between oocytes). Each oocyte is encompassed by a layer of follicle cells and a zona pellucida. Cortical alveoli rim the periphery of each oocyte. The ooplasm of the preovulatory oocytes is intensely PAS-positive (*). PAS/MY, Bar = 50 µm. ca, cortical alveoli; EVS, extravascular space; FC, follicle cells; od, oil droplet; og, oil globule; OL, ovarian lumen; y, yolk; yg, yolk globules; ZP, zona pellucida.

preovulatory oocyte and a POC after ovulation are juxtaposed (Fig. 9) so that they may be compared. The follicle cells and postovulatory follicle cells are vacuolated with a material that is PAS-positive. This is a clear, mucus-like mucomatrix observed within the ovarian lumen in the ovulating female when the ovary was dissected for histology.

The postovulatory follicle complex (POC; Fig. 10) is a complicated, folded structure that assumes many shapes after ovulation. It has a lumen that is lined by vacuolated, postovulatory follicle (POF) cells. At the point where the oocyte emerged from the follicle, these cells are joined to the epithelial cells of the germinal epithelium. Blood vessels that appear along the surface of the postovulatory follicle are actually within the postovulatory theca and are separated from the postovulatory follicle by a basement membrane that is stained indistinctly with PAS. The obliquely sectioned portion of the postovulatory follicle (POF2) illustrates the degree of vacuolization of the postovulatory follicle cells (Fig. 10, inset).

The egg and fertilization.—The egg of the Florida Bass approximates 1.2 mm in diameter. Yolk consists of numerous clear globules and, generally, a single large oil globule, although smaller oil globules may commonly be observed. Cortical alveoli encircle the apical ooplasm. There is little discernable difference in egg polarity aside from a slightly thicker ooplasm at the animal pole in comparison to the vegetal pole (Fig. 11A). PAS-positive cortical alveoli are located around the peripheral ooplasm (Fig. 11A). The egg is enclosed in the egg envelope that was the zona pellucida prior to ovulation. The surface of the egg is smooth and there are no filaments. The bi-layered egg envelope (Fig. 11B) has a small opening on its surface, the micropyle, through which a sperm can pass and fertilize the egg. After fertilization, cortical alveoli release ensues, beginning at the animal pole (Fig. 11C) which becomes scalloped in appearance due to their release. Cortical alveoli contents become a PAS-stained material in the space beneath the egg envelope (Fig. 11D, E) and also form "membranous" structures that, at least initially, attach to the inner surface of the egg envelope (Fig. 11D, arrow heads; Fig. 11E). Cortical alveoli release proceeds from the animal to the vegetal pole of the egg, over an extended period of time (Table 2). The optimal time to fix eggs of Florida Bass for the study of cortical alveoli release is between 10 to 20 seconds after activated sperm are poured upon stripped eggs.

The mechanism of fertilization allows a single sperm to enter and pass through the micropyle and fertilize the egg and trigger the release of cortical alveoli. Upon release, flattened, apparently membrane-bound, PAS-positive vacuoles appear along the inner surface of the egg envelope (Fig. 11C, D, E). In a sample 1.5 minutes after activated sperm were placed on the eggs, a micropyle with three sperm was observed (Fig. 11E, asterisk). Resolution was sufficient to observe sperm flagella (Fig. 11F). PAS-positive, cortical alveolar material covered the lower opening of the micropyle, preventing access of these sperm to the egg.

Atresia.—Given the high rate of oocyte development (primary, secondary growth, and ovulation), there is a low number of atretic oocytes. Atresia can occur during any stage of oocyte growth, even maturation. Atresia of primary growth oocytes (Fig. 12A) is indicated by fragmentation of the basophilic ooplasm. No germinal vesicle was observed.

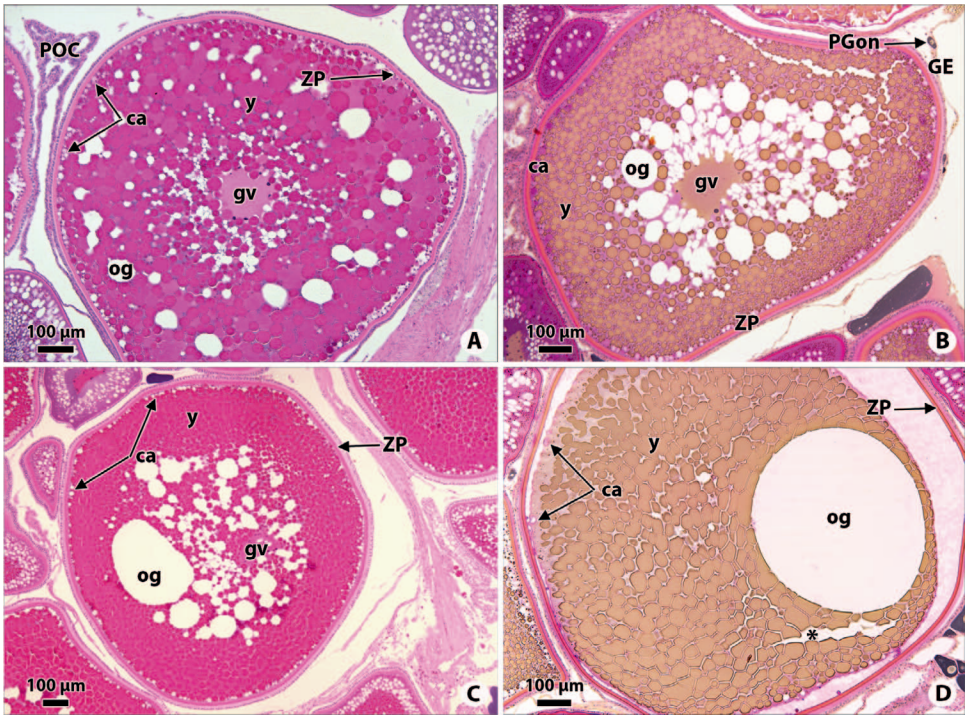


Fig. 7. *Micropterus floridanus*. (A) At the initiation of oocyte maturation, small oil droplets surrounding the germinal vesicle begin to fuse, becoming oil globules. Cortical alveoli are around the periphery of the oocyte, beneath the zona pellucida. A postovulatory follicle complex is next to the oocyte beginning maturation. H&E, Bar = 100 µm. (B) As oil globules fuse, they are more restricted to the region around the germinal vesicle and are surrounded by yolk globules, exterior to which is a layer of cortical alveoli beneath the zona pellucida. A single nucleolus, primary growth oocyte is attached to the germinal epithelium. H&E, Bar = 100 µm. (C) As oil globules continue to fuse, one becomes larger than others. As above, the oocyte has a peripheral layer of cortical alveoli beneath the zona pellucida. H&E, Bar = 100 µm. (D) A preovulatory oocyte with but one oil globule, numerous fluid yolk globules, peripheral cortical alveoli and surrounded by a zone pellucida. The fluid yolk globules are separated (*). PAS/MY, Bar = 100 µm. ca, cortical alveoli; GE, germinal epithelium; gv, germinal vesicle; og, one or more oil globules; PGon, Primary Growth Stage, one nucleolus step oocyte; POC, postovulatory follicle complex; y, yolk globules; ZP, zona pellucida.

Atresia was also observed in a small number of vitellogenic oocytes (Fig. 12B, C). These were characterized by loss of the germinal vesicle and complete breakdown and disappearance of the zona pellucida. Yolk and oil droplets became randomly intermixed. Early in atresia, yolk is present in the atretic follicle (Fig. 12B), and follicle cells become phagocytes and invade the ooplasm (Fig. 12C) to digest the oocyte contents. In late atresia, atretic oocytes (Fig. 12D) are small structures composed of follicle cell nuclei and a few vacuoles. Presumably, these end-stage atretic oocytes disappear from the ovarian stroma.

A single, preovulatory oocyte in early atresia was observed (Fig. 13A). Yolk globules were coalescing within the fluid yolk, and the zona pellucida was still intact but was becoming internalized within the atretic oocyte. Breakage of the zona pellucida was inferred because follicle cells were actively phagocytizing yolk globules at the oocyte periphery (Fig. 13B). The cortical alveoli were nearly gone from the ooplasm; only a few remained, clumped together within the fluid yolk. Fluid yolk composed most of the ooplasm (Fig. 13A). When stained for reticulin, early atretic follicles with yolk remained encompassed by a black, intact basement membrane (Fig. 13C). Follicle cell nuclei were scattered within the atretic oocyte.

DISCUSSION

Oocyte development and staging.—Oocyte development in Florida Bass progresses through six stages (Grier et al., 2009; Table 1) that are based on mitosis and meiosis. We illustrate all six stages and document comparative information on ovulation.

As typical for teleost fishes that are cystovarian, in which the ovarian lumen is continuous with the gonoduct, the ovary of the Florida Bass has a central lumen. Lamellae project into the lumen from the ovarian wall. A germinal epithelium lines the lamellar surfaces. Proliferation of oogonia occurs within the germinal epithelium where a single oogonium, or oogonia in cell nests, undergoes folliculogenesis. Within cell nests or along the germinal epithelium, oogonia enter into meiosis to become oocytes that may enter pachytene in unison, or not. Oocytes enter the primary growth stage when they have a single nucleolus and basophilic ooplasm. Folliculogenesis is completed in the One Nucleolus Step of the Primary Growth Stage (PGon), as in Common Snook (Grier, 2000) and Red Drum (Grier, 2012). Epithelial cells within the germinal epithelium become prefollicle cells when associated with oogonia, chromatin nucleolus oocytes and one nucleolus oocytes that are completing folliculogenesis, as in other fishes (Grier, 2000, 2012; Quagio-Grassiotto et al., 2011; Wildner et al., 2013).

That basement membranes gradually encompass an oocyte and its prefollicle cells during folliculogenesis is illustrated with the reticulin stain. Reticular fibers are part of the basement membrane (Mazzoni et al., 2014) that underlies the germinal epithelium and also extends around cell nests and single oocytes that are completing folliculogenesis; they isolate the developing follicle from the germinal epithelium. Reticular fibers also surround individual follicles that are composed of a single oocyte and surrounding layer of follicle

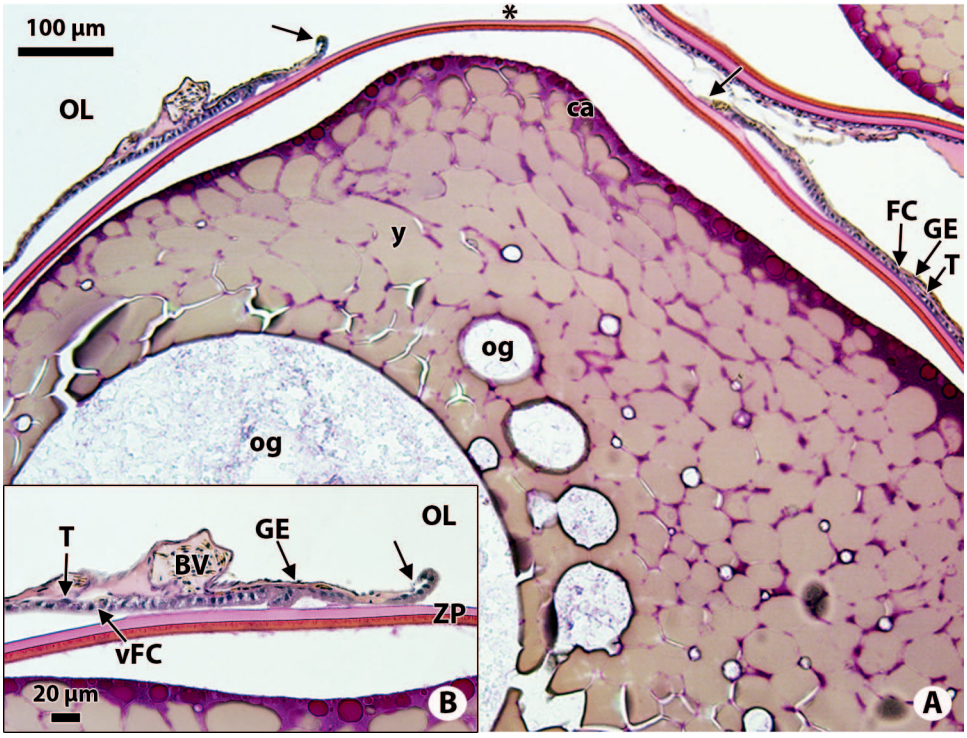


Fig. 8. *Micropterus floridanus*. (A) Ovulation is the emergence of the oocyte from the follicle, in which it developed, to become an egg or ovum. Small, unlabeled arrows point to the "break" in the germinal epithelium and follicle cells indicating the beginning of ovulation. The zona pellucida is exposed (*) to the ovarian lumen between the arrows as the follicle cells and the epithelial cells in the germinal epithelium part. A single, large oil globule is semi-surrounded by smaller oil globules, still fusing. Fluid yolk globules fill the deeply PAS-positive ooplasm. Cortical alveoli are located around the periphery of the oocyte. PAS/MY, Bar = 100 µm. (B) Enlargement of the ovulating oocyte where epithelial cells of the germinal epithelium form a continuum with follicle cells (unlabeled arrow) of the ovulating oocyte. The follicle cells are vacuolated, and thecal cells are miniscule. At the zona pellucida label, the zona pellucida contacts the ovarian lumen. PAS/MY, Bar = 20 µm. BV, blood vessels; ca, cortical alveoli; FC, follicle cells; GE, germinal epithelium; og, oil globule; OL, ovarian lumen; T, thecal cell; vFC, vacuolated follicle cells; ZP, zona pellucida.

cells. Reticular fibers were observed within the stroma and extend between follicles, apparently being an acellular, stromal connective tissue, and probably within the stromal cell connections that were observed between follicles (compare Fig. 2 and Fig. 5B). Therefore, in addition to being part of basement membranes, reticular fibers also extend between follicles, perhaps forming a loosely connected network within the lamellae.

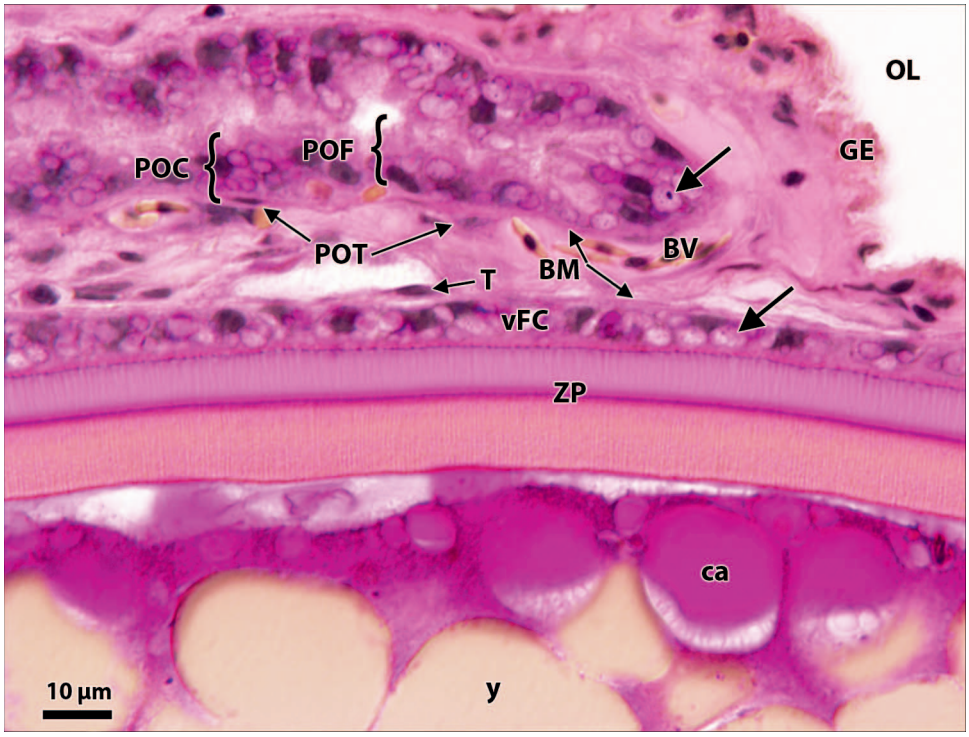


Fig. 9. *Micropterus floridanus*. A preovulatory oocyte below a postovulatory follicle complex, in which the ooplasm has peripheral cortical alveoli and yolk globules. The zona pellucida is bi-layered. The vacuolated follicle cells are similar to the postovulatory follicle cells (large arrows). Outside of the follicle cell layer is a squamous thecal cell layer. A basement membrane (arrows) separates the follicle cells from the theca. A postovulatory follicle complex is composed of a postovulatory follicle, having vacuolated cells (arrows) and the postovulatory theca. The basement membrane, which separates the postovulatory theca from the stroma, is resolved. PAS/MY, Bar = 10 µm. BM, basement membrane; BV, blood vessels; ca, cortical alveoli; GE, germinal epithelium; OL, ovarian lumen; POC, postovulatory follicle complex; POF, postovulatory follicle; POT, postovulatory theca; T, thecal cells; vFC, vacuolated follicle cells; y, yolk globules; ZP, zona pellucida.

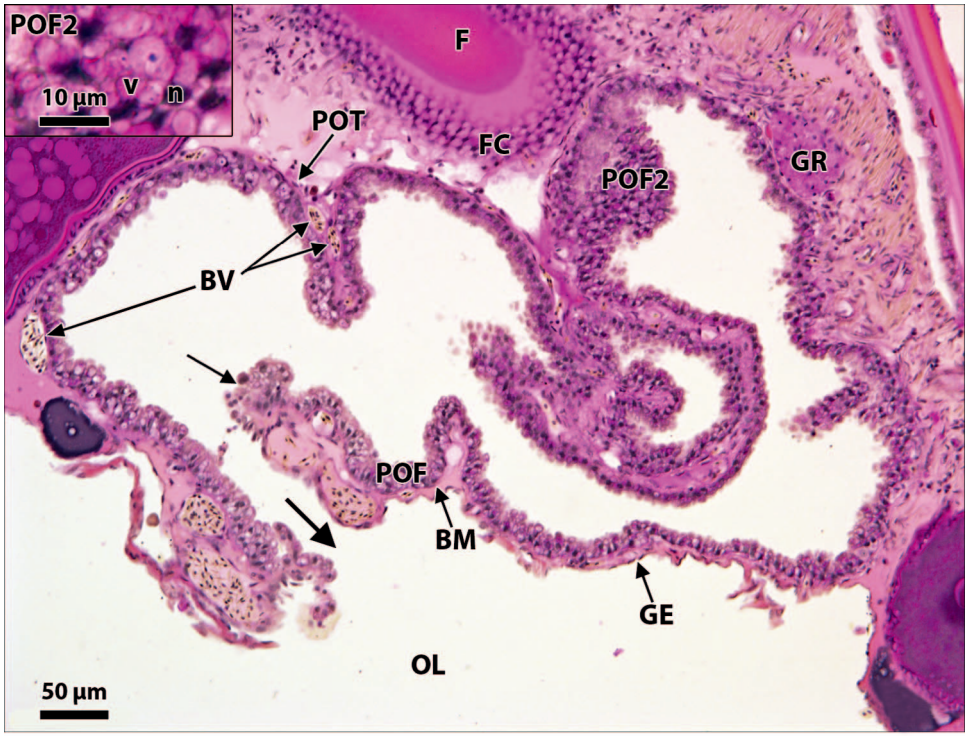


Fig. 10. *Micropterus floridanus*. A postovulatory follicle complex is composed of a postovulatory follicle and a postovulatory theca separated by a basement membrane. Blood vessels are in the postovulatory theca, and some granulomas are in the stroma. Large arrow marks the opening through which the oocyte entered the ovarian lumen to become an egg at ovulation. Small arrow indicates where the postovulatory follicle joins the germinal epithelium. PAS/MY, Bar = 50 µm. Inset: an enlargement from POF2 to illustrate vacuolated postovulatory follicle cells. PAS/MY, Bar = 10 µm. BM, basement membrane; BV, blood vessels; F, tangential section of a follicle; FC, follicle cells; GE, germinal epithelium; GR, granuloma; n, nucleolus; OL, ovarian lumen; POF, postovulatory follicle; POF2, tangential portion of POF; POT, postovulatory theca; v, PAS-positive vacuole in POF cells.

Through gene amplification (Guraya, 1986), the single, large nucleolus of the one nucleolus oocyte produces numerous, smaller nucleoli that become aligned around the germinal vesicle periphery, signifying the Perinucleolar Step (pn) within the Primary Growth Stage (PG) of oocyte development, or PGpn. A major primary growth step occurs with the appearance of PAS-positive cortical alveoli in the perinucleolar region of the ooplasm, signifying the Cortical Alveolar Step in the Primary Growth Stage, or PGca. Simultaneously, some oil droplets also appear as clear vacuoles with either H&E or PAS/MY stains. But the Oil Droplets Step is not defined until these completely surround the germinal vesicle (Grier et al., 2009). After cortical alveoli appear, small yolk granules form which indicates the beginning of vitellogenesis, the Early Secondary Growth Step of the Secondary Growth Stage, or SGe. As secondary growth continues, the oil droplets become scattered among numerous yolk granules, some of these reaching maximum diameter and, thus, signifying the late Secondary Growth Step of the Secondary Growth Stage, or SG*l*.

The Oocyte Maturation Stage begins noticeably in Florida Bass when oil droplets start to coalesce to become larger oil globules. The oocyte grows because of an uptake of water or hydrolysis (see below). When maturation is complete, the preovulatory oocyte has a single, large oil globule and possibly some smaller globules. An interesting phenomenon develops during oocyte growth. The ooplasm is initially basophilic in both H&E and PAS/MY stains, being particularly basophilic (stains with hematoxylin) in primary growth oocytes. But, during oocyte maturation, the ooplasm is basophilic when stained with H&E and becomes PAS-positive when stained with PAS/MY. The significance of this shift in staining property is unknown, yet typical of ovulating Florida Bass oocytes.

After a second meiotic arrest, the final stage of oocyte growth and maturation is ovulation. Ovulation begins when the follicle cell layer, basement membrane surrounding the oocyte, and the overlying epithelial cell layer of the germinal epithelium break to create an opening to the ovarian lumen. The oocyte can leave the follicle to become an egg or ovum. The process has been documented previously (Grier et al., 2009) and may be the same in all fishes. At ovulation, the follicle cells and the epithelial cells of the germinal epithelium, once separated by a basement membrane, become joined at the break. When the egg emerges from the follicle, the former remnants of the follicle complex, the follicle cells, basement membrane, and thecal cells, are left behind. These become a postovulatory follicle complex (POC), a compound structure that includes the postovulatory follicle (POF), the basement membrane and the postovulatory theca (POT; Grier et al., 2009, 2016; Grier, 2012).

The encompassing basement membrane around the postovulatory follicle and presence of a postovulatory theca has gone unrecognized in the fish literature: the existence of these structures, following ovulation, was unknown. They were first reported in Common Snook (Taylor et al., 1998) as a postovulatory follicle that also included the basement membrane and the postovulatory theca. We now understand that the follicle cells and the postovulatory follicle cells are derived from a germinal epithelium (Grier et al., 2016), whereas the theca is derived from stroma, which is derived from mesenchyme, a different tissue compartment. Thus, the postovulatory follicle complex (POC) is derived from two different tissue compartments separated by a basement membrane.

We report a function of the POC in Florida Bass previously unknown in any fish species. Prior to ovulation, the follicle cells are vacuolated, and the vacuole contents stain with PAS. Upon dissection and fixation of the ovary that had ovulating oocytes, we noted a clear, viscous, mucus-like fluid within the ovarian lumen. Histologically, the origin of this mucus-like fluid must be the vacuolated follicle cells (Figs. 9, 10) plus the cells within the postovulatory follicle. The function of this fluid is unknown, but it may protect the eggs immediately upon ovulation and also upon release into freshwater during spawning. We identify this mucus-like fluid as a mucomatrix that is synthesized during late

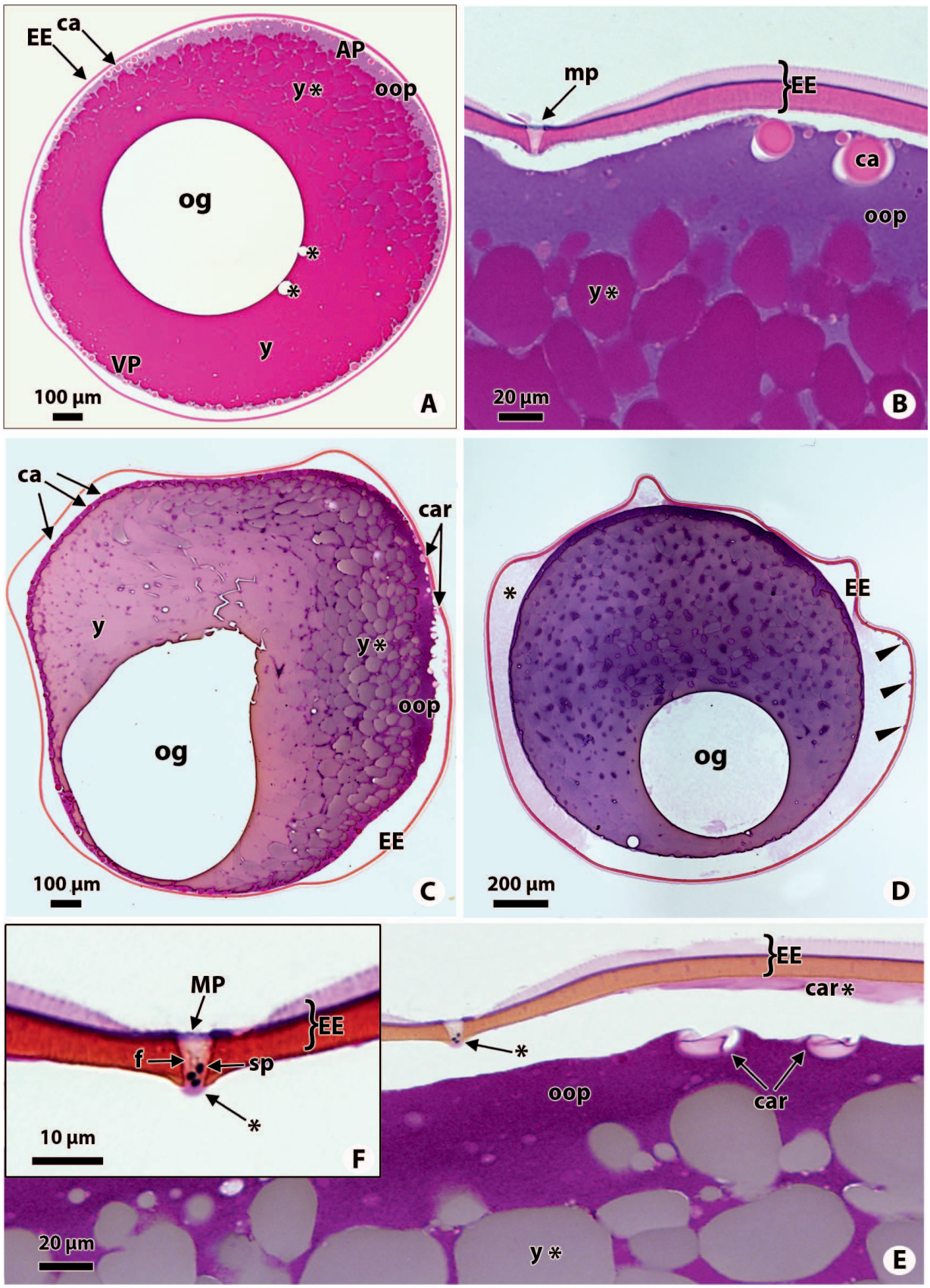


Fig. 11. *Micropterus floridanus*. (A) An unfertilized egg is encompassed by an egg envelope, has basophilic ooplasm and eosinophilic, peripheral cortical alveoli. The animal pole has slightly more ooplasm than does the vegetal pole. There is a single, large oil globule and a few smaller ones (*). Vegetal pole yolk is more homogeneous than animal pole yolk. H&E, Bar = 200 µm. (B) An infertile egg to illustrate basophilic ooplasm and distinctive, eosinophilic yolk globules and cortical alveoli at the animal pole. The egg envelope is bi-layered and has a single micropyle at the animal pole. (C) 15 to 20 seconds after fertilization, cortical alveoli begin to be released at the animal pole and result in its having a scalloped ooplasm surface in sections. Release of cortical alveoli is not yet observed at the equator or vegetal pole. Ooplasm is PAS-positive. A distinctive difference exists

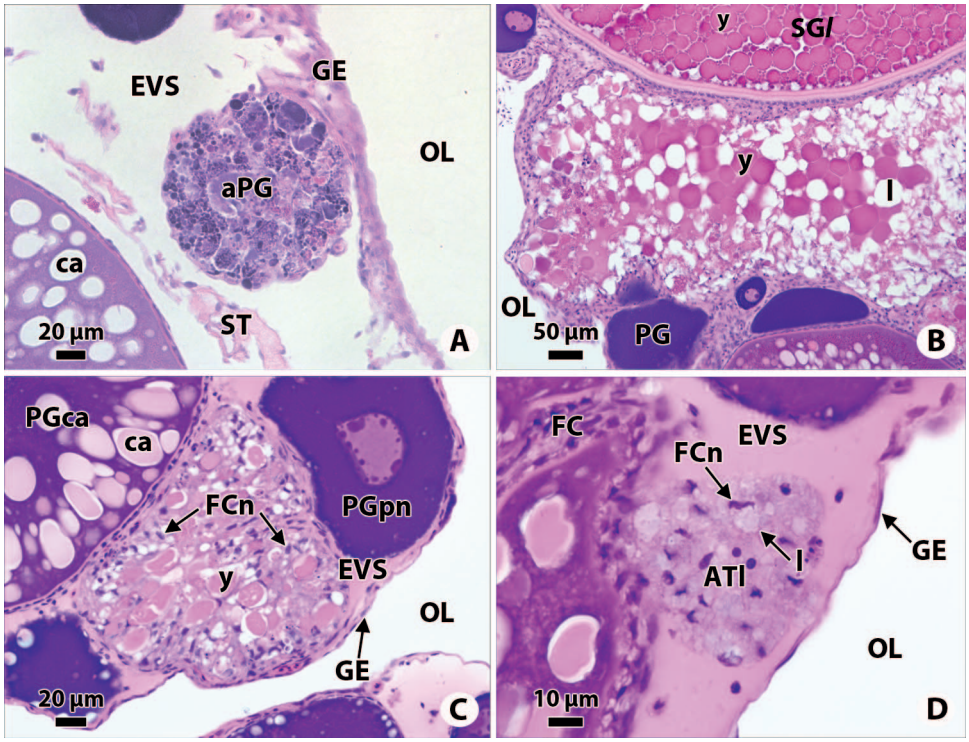


Fig. 12. *Micropterus floridanus*. (A) Atretic, primary growth oocyte. Basophilic ooplasm is fragmented. The atretic oocyte extends into the extravascular space from the germinal epithelium that borders the ovarian lumen. H&E, Bar = 20 µm. (B) Atresia of a secondary growth oocyte shows disorganized yolk globules and numerous lipid droplets. The atretic oocyte is bordered by primary growth oocytes and a late secondary growth oocyte with yolk globules. H&E, Bar = 50 µm. (C) Atresia of a secondary growth oocyte, still with yolk globules, but follicle cell nuclei have invaded the ooplasm. A primary growth, perinucleolar oocyte and one with cortical alveoli are present. H&E, Bar = 20 µm. (D) An atretic oocyte in late atresia resides in the extravascular space and is composed of only lipid vacuoles and follicle cell nuclei, at the level of resolution. A germinal epithelium separates the extra vascular space from the ovarian lumen. PAS/MY, Bar = 10 µm. aPG, atretic, Primary Growth oocyte; ATl, late atresia; ca, cortical alveoli; EVS, extravascular space; FC, follicle cells; FCn, follicle cell nuclei; GE, germinal epithelium; l, lipid vacuoles; OL, ovarian lumen; PG, Primary Growth oocyte; PGca, Primary Growth Stage, cortical alveolar step oocyte; PGpn, Primary Growth Stage, perinucleolar step oocyte; SGl, late Secondary Growth oocyte; ST, stromal cells; y, yolk globules.

secondary growth and oocyte maturation; the mucomatrix is evident in the cytoplasm of vacuolated follicle cells that surround preovulatory and ovulating oocytes.

Atresia is rare in the ovaries of reproductive Florida Bass. The first organelle to disappear from an atretic oocyte is the germinal vesicle; it was never observed in atretic oocytes. The ooplasm of atretic primary growth oocytes simply fragmented. During atresia of secondary growth oocytes, the zona pellucida disappeared, follicle cell nuclei invaded the ooplasm, and yolk globules were arranged randomly with oil droplets throughout the ooplasm. The hallmark of late atresia was a small, shrunken structure composed of scattered, persistent follicle cell nuclei that were associated with some lipid droplets. Atresia of a preovulatory oocyte was noted; the yolk globules had coalesced primarily into a fluid mass. Atresia may occur in any stage of oocyte development in Florida Bass. We note that when we observed reticulin-stained atretic oocytes, they were still encompassed by a basement membrane indicating that atresia occurred within the follicle, isolated from the encompassing thecal cells and stroma. As atresia advanced, and the atretic oocytes became noticeably smaller, it seemed logical that "liquefied" ooplasm could diffuse across the basement membrane and into the stroma from which it could be recycled. Reticulin revealed that the basement membrane of late atretic follicles was incomplete and breaking up. Obviously, since late atretic follicles were rare in ovaries of Common Snook while early atretic follicles and mid atretic follicles were common, these late atretic follicles disappear from the ovary.

Cortical alveoli release.—The timing of release of cortical alveoli following fertilization in Florida Bass appears to be

←
between globule animal pole yolk and more homogeneous vegetal pole yolk. The oocyte has a single oil globule. PAS/MY, Bar = 10 µm. (D) A fertilized egg after all cortical alveoli have been released. Remnants of cortical alveoli are against the egg envelope (arrow heads), and there is a single oil globule. Cortical alveoli contents apparently break down and form a homogeneous, granular substance between the egg and egg envelope (*). (E) Animal pole egg surface 1.5 minutes after activated sperm were poured onto swirled eggs. Cortical alveoli are still being released from the fertilized egg at the animal pole; the released contents have accumulated beneath an egg envelope. The micropyle contains three sperm which cannot pass through the lower micropyle orifice because of PAS-positive cortical alveoli release (arrow, asterisk). PAS/MY, Bar = 20 µm. (F) Enlargement of the egg envelope to illustrate the micropyle with three sperm that are blocked from passing through the micropyle by PAS-positive cortical alveoli release (arrow, asterisk). Sperm flagella are visible. PAS/MY, Bar = 10 µm. AP, animal pole; ca, cortical alveoli; car, cortical alveoli being released; car*, cortical alveoli contents against egg envelope; EE, egg envelope; f, sperm flagella; mp, micropyle; og, oil globule; oop, animal pole ooplasm; sp, sperm; VP, vegetal pole; y, vegetal pole yolk; y*, globular animal pole yolk.

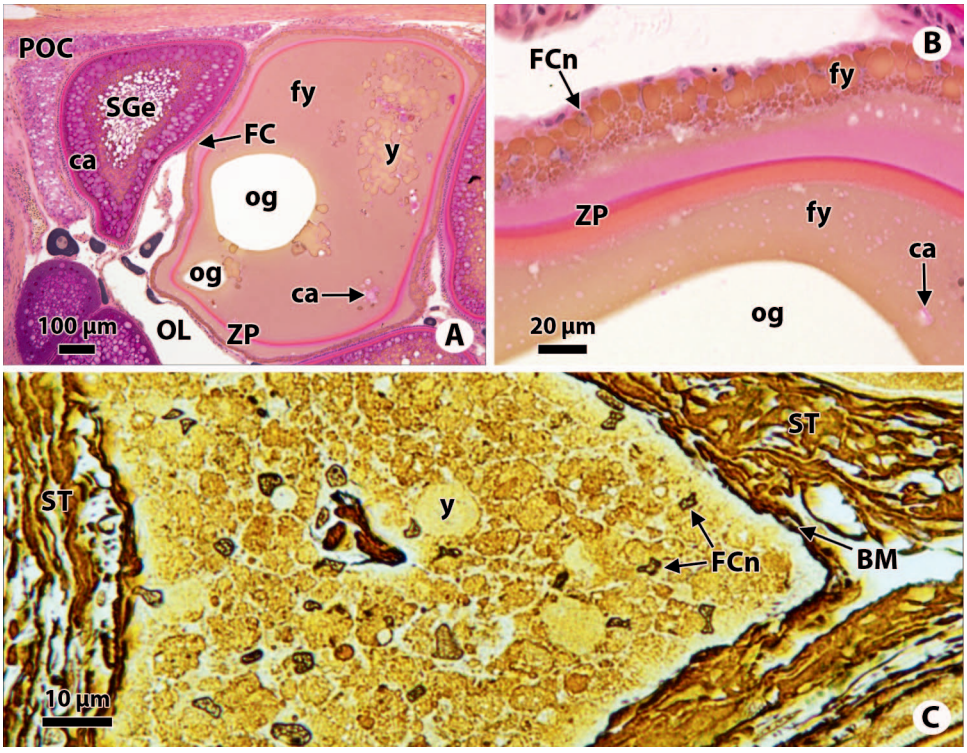


Fig. 13. *Micropterus floridanus*. (A) Atretic preovulatory oocyte. The zona pellucida is still intact, but slightly internalized, separated from the follicle cell layer that appears "granular." There are two oil globules, and cortical alveoli have largely disappeared. Those that remain are internalized. Yolk has become fluid aside from a few globules that still remain. An early secondary growth oocyte has numerous cortical alveoli around its periphery, and small yolk globules and oil droplets internal to them are seen along with a postovulatory follicle complex. PAS/MY, Bar = 100 µm. (B) Surface of the atretic, preovulatory oocyte. The follicle cells are phagocytizing yolk; their nuclei are located between ingested yolk particles. Within the ooplasm, the internalized zona pellucida, fluid yolk, cortical alveoli remnants, and the oil globule are seen. PAS/MY, Bar = 20 µm. (C) Late atresia wherein a couple of yolk globules remain in the ooplasm that is being phagocytized by follicle cells whose nuclei are scattered throughout the atretic oocyte. An intact basement membrane surrounds the atretic oocyte. Reticulin Stain, Bar = 10 µm. BM, basement membrane; ca, cortical alveoli; FC, follicle cell layer; FCn, follicle cell nuclei; fy, fluid yolk; og, oil globule(s); OL, ovarian lumen; POC, postovulatory follicle complex; SGe, early secondary growth oocyte; ST, stromal cells; y, yolk globule; ZP, zona pellucida.

unique among fishes: we observed an extended time-release sequence, occurring over minutes (Table 2). Cortical alveoli release following fertilization begins at the animal pole of the egg, spreads to the equator after two minutes in the majority of eggs, and then to the vegetal pole, with the majority of eggs in this final part of cortical alveoli release ten minutes after activated sperm were poured on the eggs. Our unreported observations with Common Snook, *Centropomus undecimalis*, indicate cortical alveoli release was completed by five seconds after activated sperm were poured on eggs at 26° to 28°C. The prolonged release of Florida Bass cortical alveoli might be due to large egg size and water temperature, around 18°C. The release of cortical alveoli is hypothesized to help prevent polyspermy in teleosts (e.g., Bazzoli and Pereira Godinho, 1994). This agrees with our observation that the micropyle was blocked by an accumulation of PAS-positive material following cortical alveoli release, preventing additional sperm from entering the perivitelline space and fertilizing the egg, which would result in an abnormal chromosome number.

Evolution of pelagic eggs.—As oocytes develop, they are first demersal, then become potentially pelagic through hydrolysis:

> "All opaque eggs sink in sea water, and all mature translucent eggs float in sea water of ordinary density. It is this property of the mature ovum that renders it *pelagic* [italics in original]; before the change occurs it is a demersal egg" (Fulton, 1898: 114).

Thus, the evolution of the actinopterygian egg is reflected in oocyte development. The most primitive living actinopterygian taxa—Polypteriformes, Acipenseriformes, and Holostei—are obligate freshwater spawners. They evolved in epicontinental seas and their freshwater margins and, even if they venture into marine waters, return to freshwater to spawn (Parenti, 2008). Their egg is mesolecithal and has semi-holoblastic cleavage (Finn and Kapoor, 2008). It contains granular, protein yolk (Cherr and Clark, 1982: fig. 1) and cannot float because it does not clear. To float, an egg must be able to take up water during oocyte maturation so that the yolk becomes fluid and clear. Teleosts have an egg that may take up water; hence, the yolk may clear. In contrast to the egg of "basal" actinopterygians, the teleost egg is telolecithal and has meroblastic cleavage (Finn and Kapoor, 2008).

This mechanism by which teleost eggs become buoyant has been known for over a century: prior to ovulation, an influx of water into the oocyte renders the cellular fluid less dense (with a lower specific gravity) than seawater (Fulton, 1898; Wallace and Selman, 1989). How this occurs has only been understood recently. Clearing occurs during oocyte maturation due to the breakdown of yolk into constituent, osmotically active, free amino acids (FAA; Fyhn et al., 1999; Finn et al., 2000; Finn and Kristoffersen, 2007). The inferred

genetic mechanism is a duplication of the yolk producing genes (Vitellogenin: VtgA and VtgB) and subsequent degradation of yolk proteins from one of the duplicated genes (VtgB); the breakdown of yolk into FAA results in a net influx of water from the follicle to the oocytes such that they double or more in size prior to ovulation (Finn and Kristoffersen, 2007). Water crosses past or through the surrounding egg cellular or intercellular spaces and acellular layer (theca, basement membrane, and follicle cells) by osmosis and dilutes the yolk. With osmotically active, free amino acids, the yolk clears and the egg can float (Finn and Kristoffersen, 2007). This simple mechanism allowed fishes to move from a largely coastal freshwater and euryhaline environment to the seas an estimated 100 million years ago (Fyhn et al., 1999; Finn and Kristoffersen, 2007).

Yolk breakdown, or clearing, evolved initially such that the resulting pool of free amino acids could be readily taken up and subsequently used by developing fish larvae for growth before they developed the enzymes necessary to break down yolk to use during larval development (see Rønnestad et al., 1999; Finn and Fyhn, 2010). This hypothesis suggests that yolk breakdown evolved in concert with larval development in pelagic marine teleosts. On this topic, Fulton (1898: 90) wrote:

> "A knowledge of the nature of the yolk in pelagic eggs…explains the gradual sinking of the larvae after they are hatched… It is in virtue of the watery yolk of low specific gravity that the egg floats, and as this becomes used up in the growth of the little fish, to which it is attached, and transformed into denser tissues, the specific gravity of the whole is increased, until it exceeds that of the sea water in which it is immersed. Hence the general rule that pelagic eggs are obtained in the surface layers of the sea, while the larvae are found most abundantly towards the bottom and the middle layers. The expansion of the pelagic ovum at maturation will also be afterwards shown to be correlated in certain important respects with the life-history of the species."

With such a mechanism, coastal and probably euryhaline fishes might have had eggs with fluid yolk that facilitated larval development. Secondarily, fluid yolk enabled eggs to be buoyant in saltwater. Thereby, a mechanism that evolved initially as part of larval fish development became the underlying mechanism that led to the evolution of pelagic eggs.

Pelagic versus demersal eggs in fish classification.—The clear, fluid yolk of Florida Bass preovulatory oocytes consists of numerous globules. These are quite unlike the large "globules" that subdivide fluid yolk in Common Snook preovulatory oocytes and eggs (Taylor et al., 1998; Grier, 2000; Rhody et al., 2014), in the Northern Anchovy, *Engraulis mordax* (see Hunter and Macewicz, 1985), or in the continuous fluid mass of the Red Drum (Grier, 2012) and other fishes that produce pelagic eggs. Further, the Florida Bass egg is large and has a relatively thick egg envelope. The egg envelope is externally sticky which makes the egg adherent (demersal); otherwise, it would be free (pelagic). It shares these egg characters with its close relative the diminutive, freshwater *Elassoma* (Betancur-R. et al., 2017). For example, *Elassoma okefenokee* has a large, spherical egg with a diameter of 2 to 2.2 mm (Bohlen and Nolte, 1999: fig. 5). It has a large oil globule of 110 to 120 μm inside a yolk sphere of about 750 μm. Between the egg envelope and the yolk is the perivitelline jelly, an egg coating that swells when the eggs are deposited on a substrate. The relationship between the so-called perivitelline jelly of *E. okefenokee* and the PAS-positive material released by the cortical alveoli of *M. floridanus* (Fig. 11D) is unknown. The eggs of *E. okefenokee* are "…slightly adhesive and hold by mechanically fixing the egg in …interspaces of the dense vegetation during the swelling process. Without the surrounding layers, the egg would fall out of the substrate…" (J. Bohlen, pers. comm, 31 January 2013). Species of *Elassoma* do not construct a nest, although they always spawn near submerged vegetation (Walsh and Burr, 1984).

The eggs of the Florida Bass and *Elassoma* may be characterized as modified pelagic eggs that are functionally demersal. The pelagic characters of the eggs reflect the close phylogenetic relationship of these basses to marine perciforms (e.g., Betancur-R. et al., 2017). Egg characters have been used to diagnose higher taxa, such as the Atherinomorpha, *sensu* Rosen and Parenti (1981: 20), which has "A large demersal egg with long adhesive and short filaments and many lipid globules that coalesce at the vegetal pole." Eggs of the atherinomorph cyprinodontiform *Fundulus heteroclitus* (see Kuchnow and Scott, 1977) are functionally and morphologically demersal: they have a thick egg envelope and elaborate filaments by which fertilized eggs attach to the substrate until hatching.

The atherinomorphs were classified in the higher taxon Ovalentaria in recent molecular phylogenetic analyses (e.g., Wainwright et al., 2012; Betancur-R. et al., 2017). The name Ovalentaria Smith and Near (in Wainwright et al., 2012: 1001), was coined to reflect the "…characteristic demersal, adhesive eggs with chorionic [egg envelope] filaments." Mugilids, the mullets, are also classified in the Ovalentaria, yet have typical small, buoyant, pelagic eggs, without filaments (Shabanipour and Heidari, 2004). Also, there are non-ovalentarian fishes, such as the gobioid *Micropercops swinhonis* (e.g., Iwata et al., 2001), that have demersal eggs with filaments.

The character, or character complex, of a demersal egg with adhesive filaments may diagnose a higher taxon of percomorph fishes, but that group has yet to be proposed. More precise descriptions of egg characters, such as the number of layers in the egg envelope or the length and distribution of the attachment filaments, across a broader range of taxa are needed to test hypotheses of homology of these traits.

Conclusion.—Florida Bass oocyte development follows precisely a pattern that has been documented for marine perciform fishes that produce positively buoyant, pelagic, non-adhesive eggs, such as the Red Drum (Grier, 2012) and the Common Snook (Rhody et al., 2014). Yet the eggs of the freshwater Florida Bass are demersal and adhesive, laid in nests and guarded by males. Classification of a fish egg as pelagic or demersal should include morphology as well as function. Eggs of all of these species have clear yolk. The clearing of yolk due to the uptake of water during oocyte development in actinopterygians, as they progress from a demersal to a potentially pelagic phase, may be a general mechanism of oocyte maturation in teleost fishes (Fulton, 1898).

ACKNOWLEDGMENTS

We thank the many people who contributed to this work. For their boating skills and other assistance during field collections, we are indebted to Cheree Steward, Brandon Thompson, Bill Johnson, and Andrew Schaefer of the Florida Fish and Wildlife Conservation Commission. Noretta Perry,

Meredith Grinnell, and Yvonne Waters provided invaluable assistance with histology. Joerg Bohlen, Casey Dillman, Mike Matthews, and Helen Wimer provided references and other valuable information or assistance. Our thanks go to Mote Aquaculture Research Park for access to their Olympus microscope and digital camera used to take the micrographs.

LITERATURE CITED

Ahlstrom, E. H., and H. G. Moser. 1976. Eggs and larvae of fishes and their role in systematic investigations and in fisheries. Revue des Travaux de l'Institut de Pêches Maritime 40:379–398.

Ahlstrom, E. H., and H. G. Moser. 1980. Characters useful in identification of pelagic marine fish eggs. California Cooperative Oceanic Fisheries Investigations Report 21: 121–131.

Bailey, R. M., and C. L. Hubbs. 1949. The black basses (*Micropterus*) of Florida, with description of a new species. University of Michigan, Museum of Zoology, Occasional Papers 516:1–40.

Barthel, B. L., M. S. Allen, W. F. Porak, and J. Kerns. 2015. Florida Bass *Micropterus floridanus* (LeSueur, 1822), p. 43–53. *In:* Black Bass Diversity: Multidisciplinary Science for Conservation. M. D. Tringali, J. M. Long, T. W. Birdsong, and M. S. Allen (eds.). American Fisheries Society Symposium 82, Bethesda, Maryland.

Bazzoli, N., and H. Pereira Godinho. 1994. Cortical alveoli in oocytes of freshwater neotropical freshwater fish. Bolletino di Zoologia 61(4):301–308.

Berois, N., A. Arezo, and N. G. Papa. 2011. Gamete interactions in teleost fish: the egg envelope. Basic studies and perspectives as environmental biomonitor. Biological Research 44:119–124.

Betancur-R., R., E. O. Wiley, G. Arratia, A. Acero, N. Bailly, M. Miya, G. Lecointre, and G. Ortí. 2017. Phylogenetic classification of bony fishes. BMC Evolutionary Biology 17: 162.

Bohlen, J., and A. Nolte. 1999. Zur Fortpflanzungsbiologie der Familie Elassomatidae (Zwergschwarzbarsche), p. 69–78. *In*: Fortpflanzungsbiologie der Aquarienfische (2). R. Riehl and H. Greven (eds.). Schmettkamp-Verlag, Bornheim.

Breder, C. M., Jr., and D. E. Rosen. 1966. Modes of Reproduction in Fishes. Natural History Press, New York.

Bruno, N. A., R. W. Gregory, and H. L. Schramm, Jr. 1990. Nest sites used by radio-tagged Largemouth Bass in Orange Lake, Florida. North American Journal of Fisheries Management 10:80–84.

Carr, M. H. 1942. The breeding habits, embryology and larval development of the large-mouthed black bass in Florida. Proceedings of the New England Zoological Club 20:43–77.

Cherr, G. N., and W. H. Clark, Jr. 1982. Fine structure of the envelope and micropyles in the eggs of the white sturgeon, *Acipenser transmontanus* Richardson. Development Growth and Differentiation 24:341–352.

Chew, R. L. 1974. Early life history of the Florida Largemouth Bass. Florida Game and Fresh Water Fish Commission, Fishery Bulletin 7, Project Report F-24-R Tallahassee, Florida.

Chew, R. L. 1975. The Florida Largemouth Bass, p. 450–458. *In*: Black Bass Biology and Management. R. H. Stroud and H. Clepper (eds.). Sport Fishing Institute, Washington, D.C.

Clugston, J. P. 1966. Centrarchid spawning in the Florida Everglades. Quarterly Journal of the Florida Academy of Science 29:137–143.

Crumpton, J. E., S. L. Smith, and E. J. Moyer. 1977. Spawning of year class zero largemouth bass in hatchery ponds. Florida Scientist 40:125–129.

Finn, R. N., and H. J. Fyhn. 2010. Requirement for amino acids in ontogeny of fish. Aquaculture Research 41:684–716.

Finn, R. N., H. J. Fyhn, B. Norberg, J. Munholland, and M. Reith. 2000. Oocyte hydration as a key feature in the adaptive evolution of teleost fishes to seawater, p. 289–291. *In:* Proceedings of the 6th International Symposium on Reproductive Physiology of Fish. Institute of Marine Research and University of Bergen, 4–9 July 1999, B. Norberg et al. (eds.). University of Bergen, Norway.

Finn, R. N., and B. G. Kapoor (Eds.). 2008. Fish Larval Physiology. Science Publishers, Enfield, New Hampshire.

Finn, R. N., J. Kolarevic, H. Kongshaug, and F. Nilsen. 2009. Evolution and differential expression of a vertebrate vitellogenin gene cluster. BMC Evolutionary Biology 9:2.

Finn, R. N., and B. A. Kristoffersen. 2007. Vertebrate vitellogenin gene duplication in relation to the "3r hypothesis": correlation to the pelagic egg and the oceanic radiation of teleosts. PLOS ONE 2:e169.

França, G. F., H. J. Grier, and I. Quagio-Grassiotto. 2010. A new vision of the origin and the oocyte development in the Ostariophysi applied to *Gymnotus sylvius* (Teleostei, Gymnotiformes). Neotropical Ichthyology 84:787–804.

Fulton, T. W. 1898. On the growth and maturation of the ovarian eggs of teleostean fishes. Sixteenth Annual Report of the Fishery Board for Scotland. Part III. Scientific Investigations, p. 88–134.

Fyhn, H. J., R. N. Finn, M. Reith, and B. Norberg. 1999. Yolk protein hydrolysis and oocyte free amino acids as key features in the adaptive evolution of teleost fishes to seawater. Sarsia 84:451–456.

Grier, H. J. 2000. Ovarian germinal epithelium and folliculogenesis in the common snook, *Centropomus undecimalis*. Journal of Morphology 243:265–281.

Grier, H. J. 2012. Development of the follicle complex and oocyte staging in red drum, *Sciaenops ocellatus* Linnaeus, 1776 (Perciformes, Sciaenidae). Journal of Morphology 73: 801–829.

Grier, H. J., M. C. Uribe, F. L. Lo Nostro, S. D. Mims, and L. R. Parenti. 2016. Conserved form and function of the germinal epithelium through 500 million years of vertebrate evolution. Journal of Morphology 277:1014–1044.

Grier, H. J., M. C. Uribe, and R. Patiño. 2009. The ovary, folliculogenesis, and oogenesis in teleosts, p. 25–84. *In:* Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes), Vol. 8A. B. G. M. Jamieson (ed.). Science Publishers, Enfield, New Hampshire.

Guraya. S. S. 1986. The Cell and Molecular Biology of Fish Oogenesis. S. Karger, Basel.

Hunter, J. R., and B. J. Macewicz. 1985. Rates of atresia in the ovary of captive and wild northern anchovy, *Engraulis mordax*. Fishery Bulletin 83:119–136.

Iwata, A., H. Sakai, K. Shibukawa, and S. R. Jeon. 2001. Developmental characteristics of a freshwater goby, *Micropercops swinhonis*, from Korea. Zoological Science 18:91–97.

Kassler, T. W., J. B. Koppelman, T. J. Near, C. B. Dillman, J.M. Levengood, D. L. Swofford, J. L. VanOrman, J. E. Claussen, and D. P. Philipp. 2002. Molecular and morphological analyses of the black basses: implications

for taxonomy and conservation, p. 292–322. *In:* Black Bass: Ecology, Conservation, and Management. D. P. Philipp and M. S. Ridgway (eds.). American Fisheries Society Symposium 31, Bethesda, Maryland.

Kuchnow, K. P., and J. R. Scott. 1977. Ultrastructure of the chorion and its micropyle apparatus in the mature *Fundulus heteroclitus* (Walbaum) ovum. Journal of Fish Biology 10:197–201.

Mazzoni, T. S., H. J. Grier, and I. Quagio-Grassiotto. 2014. Male gonadal differentiation and the paedomorphic evolution of the testis in Teleostei. Anatomical Record 297:1137–1162.

McDowell, E., and B. Trump. 1976. Histological fixatives for diagnostic light and electron microscopy. Archives of Pathology and Laboratory Medicine 100:405–414.

Near, T. J., T. W. Kassler, J. B. Koppelman, C. B. Dillman, and D. P. Philipp. 2003. Speciation in North American black basses, *Micropterus* (Actinopterygii: Centrarchidae). Evolution 57:1610–1621.

Page, L. M., H. Espinosa-Pérez, L. T. Findley, C. R. Gilbert, R. N. Lea, N. E. Mandrak, R. L. Mayden, and J. S. Nelson. 2013. Common and Scientific Names of Fishes from the United States, Canada, and Mexico. Seventh edition. American Fisheries Society, Special Publication 34, Bethesda, Maryland.

Parenti, L. R. 2008. Life history patterns and biogeography: an interpretation of diadromy in fishes. Annals of the Missouri Botanical Garden 95:232–247.

Parenti, L. R., H. J. Grier, and M. C. Uribe. 2015. Reproductive biology of *Chlorophthalmus agassizi* Bonaparte, 1840 (Teleostei: Aulopiformes: Chlorophthalmidae) as revealed through histology of archival museum specimens. Copeia 103:821–837.

Parenti, L. R., F. Lo Nostro, and H. J. Grier. 2010. Reproductive histology of *Tomeurus gracilis* Eigenmann, 1909 (Teleostei: Atherinomorpha: Poeciliidae) with comments on evolution of viviparity in atherinomorph fishes. Journal of Morphology 271:1399–1406.

Porter, M. D. 1997. Oocyte maturation during hormone induced spawning in Largemouth Bass, *Micropterus salmoides*. Journal of Applied Aquaculture 7:19–27.

Puchtler, H., and F. W. Waldrop. 1978. Silver impregnation methods for reticulin fibers and reticulin: a re-investigation of their origins and specificity. Histochemistry 57:177.

Quagio-Grassiotto, I., H. Grier, T. S. Mazzoni, R. H. Nóbrega, and J. P. A. Amorim. 2011. Activity of the ovarian germinal epithelium in the freshwater catfish, *Pimelodus maculatus* (Teleostei: Ostariophysi: Siluriformes): germline cysts, follicle formation and oocyte development. Journal of Morphology 272:1290–1306.

Quintero-Hunter, I., H. J. Grier, and M. Muscato. 1991. Enhancement of histological detail using metanil yellow as counterstain in periodic acid/Schiff's hematoxylin staining of glycol methacrylate tissue sections. Biotechnic and Histochemistry 66:169–172.

Rhody, N. R., C. L. Neidig, H. J. Grier, K. L. Main, and H. Migaud. 2014. Assessing reproductive condition in captive and wild common snook stocks: a comparison between the wet mount technique and histological preparations. Transactions of the American Fisheries Society 142:979–988.

Rogers, M. W., and M. S. Allen. 2009. Exploring the generality of recruitment hypotheses for largemouth bass along a latitudinal gradient of Florida lakes. Transactions of the American Fisheries Society 138:23–37.

Rønnestad, I., A. Thorsen, and R. N. Finn. 1999. Fish larval nutrition: a review of recent advances in the roles of amino acids. Aquaculture 177:201–216.

Rosen, D. E., and L. R. Parenti. 1981. Relationships of *Oryzias*, and the groups of atherinomorph fishes. American Museum Novitates 2719:1–25.

Shabanipour, N., and B. Heidari. 2004. A histological study of the zona radiata during late oocyte developmental stages in the Caspian Sea mugilid, *Liza aurata* (Risso 1810). Brazilian Journal of Morphological Science 21:191–195.

Shaw, S. 2014. Reproduction and recruitment of Florida bass *Micropterus floridanus*. Unpubl. Ph.D. diss., University of Florida, Gainesville, Florida.

Taylor, R. G., H. J. Grier, and J. A. Whittington. 1998. Spawning rhythms of common snook in Florida. Journal of Fish Biology 53:502–520.

U.S. Department of the Interior, Fish and Wildlife Service, and U.S. Department of Commerce, U.S. Census Bureau. 2006a. National Survey of Fishing, Hunting and Wildlife-Associated Recreation.

U.S. Department of the Interior, Fish and Wildlife Service, and U.S. Department of Commerce, U.S. Census Bureau. 2006b. National Survey of Fishing, Hunting and Wildlife-Associated Recreation–Florida Report.

Vidal, B. C. 1988. Histochemical and anisotropical properties characteristics of silver impregnation: the differentiation of reticulin fibers from the other interstitial collagens. Zoologische Jahrbücher. Abteilung für Anatomie und Ontogenie der Tiere 117:485–494.

Wainwright, P. C., W. L. Smith, S. A. Price, K. L. Tang, J. S. Sparks, L. A. Ferry, K. L. Kuhn, R. I. Eytan, and T. J. Near. 2012. The evolution of pharyngognathy: phylogenetic and functional appraisal of the pharyngeal jaw key innovation in labroid fishes and beyond. Systematic Biology 61:1001–1027.

Wallace, R., and K. Selman. 1989. Cellular and dynamic aspects of oocyte growth in teleosts. American Zoologist 21:325–343.

Walsh, S. J., and B. M. Burr. 1984. Life history of the banded pygmy sunfish, *Elassoma zonatum* Jordan (Pisces: Centrarchidae), in western Kentucky. Bulletin of the Alabama Museum of Natural History 8:31–52.

Wildner, D. D., H. J. Grier, and I. Quagio-Grassiotto. 2013. Female germ cell renewal during the annual reproductive cycle in ostariophysians [sic] fish. Theriogenology 79:709–724.

Yokogawa, K., K. Nakai, and K. Fujita. 2005. Mass introduction of Florida Bass, *Micropterus floridanus,* into Lake Biwa, Japan, suggested by recent dramatic genomic change. Aquaculture Science 53:145–155.