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

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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; Crompton 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 (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 Stage [previtellogenesis] and (4) Secondary Growth Stage [previtellogenesis].
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 (SGl) is italicized to distinguish the letter “l” from the number “1.”

<table>
<thead>
<tr>
<th>Stages</th>
<th>Steps</th>
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<tbody>
<tr>
<td>1. Oogonia Proliferate (OP)</td>
<td>Frequently form cell nests</td>
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<tr>
<td>2. Chromatin Nucleolus (CN)</td>
<td>Leptotene (CNI)</td>
</tr>
<tr>
<td></td>
<td>Zygotene (CNz)</td>
</tr>
<tr>
<td></td>
<td>Pachytene (CNP)</td>
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<tr>
<td>3. Primary Growth (PG)</td>
<td>Early Diplotene (CNed)</td>
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<tr>
<td></td>
<td>One-nucleolus (P Gon)</td>
</tr>
<tr>
<td>4. Secondary Growth (SG)</td>
<td>Multiple nucleoli (P Gm)</td>
</tr>
<tr>
<td></td>
<td>Perinucleolar (PGpn)</td>
</tr>
<tr>
<td></td>
<td>Circumnucleolar oil droplets (PGod)</td>
</tr>
<tr>
<td></td>
<td>Cortical alveolar (PCA)</td>
</tr>
<tr>
<td>5. Oocyte Maturation (OM)</td>
<td>Early secondary growth (SGe) or Early yolked oocytes</td>
</tr>
<tr>
<td></td>
<td>Late secondary growth (SGl) or Late yolked oocytes</td>
</tr>
<tr>
<td></td>
<td>Full-grown oocyte (SGfg)</td>
</tr>
<tr>
<td></td>
<td>Eccentric germinal vesicle (OMegv)</td>
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<tr>
<td>6. Ovulation (OV)</td>
<td>Full-grown oocyte migration to animal pole (OMgvm)</td>
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<tr>
<td></td>
<td>Germinal vesicle breakdown (OMgb)</td>
</tr>
<tr>
<td></td>
<td>Meiosis resumes; 2nd arrest (OMmr)</td>
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<tr>
<td></td>
<td>Oocyte emerges from follicle, becomes an egg</td>
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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 are functionally demersal in contrast to the positively buoyant, pelagic eggs typical of marine perciforms (Fulton, 1898; Ahlstrom and Moser, 1980; Fyhnn 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.
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.
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. 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-
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...
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; Fig. 5). There is also a concomitant size difference between early

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**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 = 10μm. (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 break-up of the Balbiani body. RET, Bar = 10μm. (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 = 100μm. 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.

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**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 = 50μm. 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.
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
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.
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 gonaduct, 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...
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. 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.

**Fig. 9.** *Micropterus floridanus.* A preovulatory oocyte below a post-ovulatory 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.
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 SGes. 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 SGl.

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 prevoluntary 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...
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; 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.
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—Polypteroiformes, Acienceriformes, 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 teleolecithal 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 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 Maciewicz, 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 groups to bony 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 gobiod M. 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 perciform 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).

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LITERATURE CITED


