

**Abstract**—This study examined the sexual differentiation and reproductive dynamics of striped mullet (*Mugil cephalus* L.) in the estuaries of South Carolina. A total of 16,464 specimens were captured during the study and histological examination of sex and maturity was performed on a subsample of 3670 fish. Striped mullet were sexually undifferentiated for the first 12 months, began differentiation at 13 months, and were 90% fully differentiated by 15 to 19 months of age and 225 mm total length (TL). The defining morphological characteristics for differentiating males was the elongation of the protogonial germ tissue in a corradiating pattern towards the center of the lobe, the development of primary and secondary ducts, and the lack of any recognizable ovarian wall structure. The defining female characteristics were the formation of protogonial germ tissue into spherical germ cell nests, separation of a tissue layer from the outer epithelial layer of the lobe-forming ovarian walls, a tissue bud growing from the suspensory tissue that helped form the ovary wall, and the proliferation of oogonia and oocytes. Sexual maturation in male striped mullet first occurred at 1 year and 248 mm TL and 100% maturity occurred at age 2 and 300 mm TL. Female striped mullet first matured at 2 years and 291 mm total length and 100% maturity occurred at 400 mm TL and age 4. Because of the open ocean spawning behavior of striped mullet, all stages of maturity were observed in males and females except for functionally mature females with hydrated oocytes. The spawning season for striped mullet recruiting to South Carolina estuaries lasts from October to April; the majority of spawning activity, however, occurs from November to January. Ovarian atresia was observed to have four distinct phases. This study presents morphological analysis of reproductive ontogeny in relation to size and age in South Carolina striped mullet. Because of the length of the undifferentiated gonad stage in juvenile striped mullet, previous studies have proposed the possibility of protandric hermaphroditism in this species. The results of our study indicate that striped mullet are gonochoristic but capable of exhibiting nonfunctional hermaphroditic characteristics in differentiated mature gonads.

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## Sexual differentiation and gonad development in striped mullet (*Mugil cephalus* L.) from South Carolina estuaries\*

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The striped mullet (*Mugil cephalus* L.) is distributed circumglobally in tropical and semitropical waters between latitudes 42°N and 42°S (Thomson, 1963; Rossi et al., 1998). Even though considered a marine species, striped mullet are euryhaline and can be found year round throughout the full range of estuarine salinities in the southeastern United States (Jacot, 1920; Anderson, 1958). Striped mullet are important throughout the world for commercial fisheries and aquaculture. In the southeastern United States there are large-scale commercial fisheries for striped mullet in North Carolina and Florida. South Carolina and Georgia have much more limited landings (NMFS<sup>1</sup>).

The commercial effort in the southeastern United States targets "roe" fish (fish containing roe) during the fall spawning migration. Throughout the rest of the year mullet are fished commercially for human consumption (particularly the west coast of Florida) and bait (Anderson, 1958). Striped mullet have a significant economic impact in the southeast where they represented a landings value of 16.4 million dollars from 1994 to 2000 (NMFS<sup>1</sup>). Striped mullet landings in the Gulf of Mexico were significantly higher with a landings value of 86.2 million dollars for the same time period. Striped mullet are also one of the most important forage fishes that occur in the estuaries of the southeast and represent a significant food source for upper level piscivores (Wenner et al.<sup>2</sup>).

General information on the biology of striped mullet has been well documented (Jacot, 1920; Anderson, 1958; Thomson, 1963, 1966; Chubb et al., 1981) but limited information is available on the reproductive biology of wild populations (Anderson, 1958; Stenger, 1959; Greeley et al., 1987; Render et al., 1995). There is a large body of work concerning striped mullet reproduction in aquaculture but many of these studies have concentrated on females by using artificial manipulation of the reproductive cycle. Although the maturation process of oocytes may be the same as that in wild striped mullet, the environment and conditions under which maturation occurred in these studies was artificial (Shehadeh et al., 1973; Kuo et al., 1974; Pien and Liao, 1975; Kelly, 1990; Tamaru et al., 1994; Kuo, 1995). This lack of in-

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<sup>1</sup> NMFS (National Marine Fisheries Service). 2001. Unpubl. data. Statistics and Economic Division, 1315 East-West Highway, Silver Spring, Md. 20910. <http://www.st.nmfs.gov/st1/index.html>.

<sup>2</sup> Wenner, C. A., W. A. Roumillat, J. E. Moran, M. B. Maddox, L. B. Daniel, and J. W. Smith. 1990. Investigations on the life history and population dynamics of marine recreational fishes in South Carolina, part 1, p. 2–22. Completion reports, Project F-37, Charleston, and Project F-31, Brunswick. South Carolina Marine Resources Research Institute, P.O. Box 12559 Charleston, S.C. 29422.

formation on reproductive biology is surprising given the worldwide importance of mullet. In particular, there have been very few studies where sexual differentiation of immature striped mullet has been examined in conjunction with histological confirmation of maturity stage in reproductively capable adults. One notable exception was the work of Stenger (1959), who although thorough in histological confirmation of the male and female developmental stages in relation to length, did not take age into consideration at differentiation or maturity. More recent studies (Chang et al., 1995; Chang et al., 1999) have examined gonad histology and plasma sex steroids during sex differentiation in young-of-the-year striped mullet up to 12 months old, but these studies did not provide any detail on fish length during development and differentiation. Other studies have examined oocyte development and relative fecundity for the reproductive assessment of female striped mullet but did not examine reproductive development in males or take into consideration an independent confirmation of fish age (Greeley et al., 1987; Render et al., 1995). Few studies have described the process of spermatogenesis in striped mullet because most efforts on the propagation and enhancement of striped mullet reproduction have concentrated on female development because of their commercial value. Grier (1981) used striped mullet in describing the cellular organization of testes and spermatogenesis as a model for synchronously spawning fishes but did not describe size and age in relation to spermatogenesis.

Striped mullet are considered isochronal spawning fishes (Greeley et al., 1987; Render et al., 1995). There are only a few observations of offshore spawning activity (Arnold and Thompson, 1958), and eggs and larvae have rarely been collected offshore (Anderson, 1958; Finucane et al., 1978; Collins and Stender, 1989). Collins and Stender (1989) concluded that striped mullet spawn in and around the edge of the continental shelf off the coasts of North Carolina, South Carolina, Georgia, and the east coast of Florida (an area often referred to as the South Atlantic Bight), but may also spawn outside the South Atlantic Bight (SAB). They also indicated a protracted spawning season that extended from October to April. This contrasts with the estimated spawning season from previous studies (2–5 months from November through March) (Jacot, 1920; Broadhead, 1956; Anderson, 1958; Arnold and Thompson, 1958; Stenger, 1959; Dindo and MacGregor, 1981; Greeley et al., 1987; Render et al., 1995; Hettler et al., 1997). Female mullet were thought to mature at three years of age at a size of 230 to 350 mm standard length (Thomson, 1951, 1963; Greeley et al., 1987).

This study had three purposes: 1) to determine at what size and age striped mullet become fully sexually differentiated and to describe the morphological characteristics of sexual differentiation in both male and female striped mullet; 2) to determine the size and age at first maturity for each sex; and 3) to describe the timing and process of gametogenesis in relation to size and age in both males and females in order to provide a

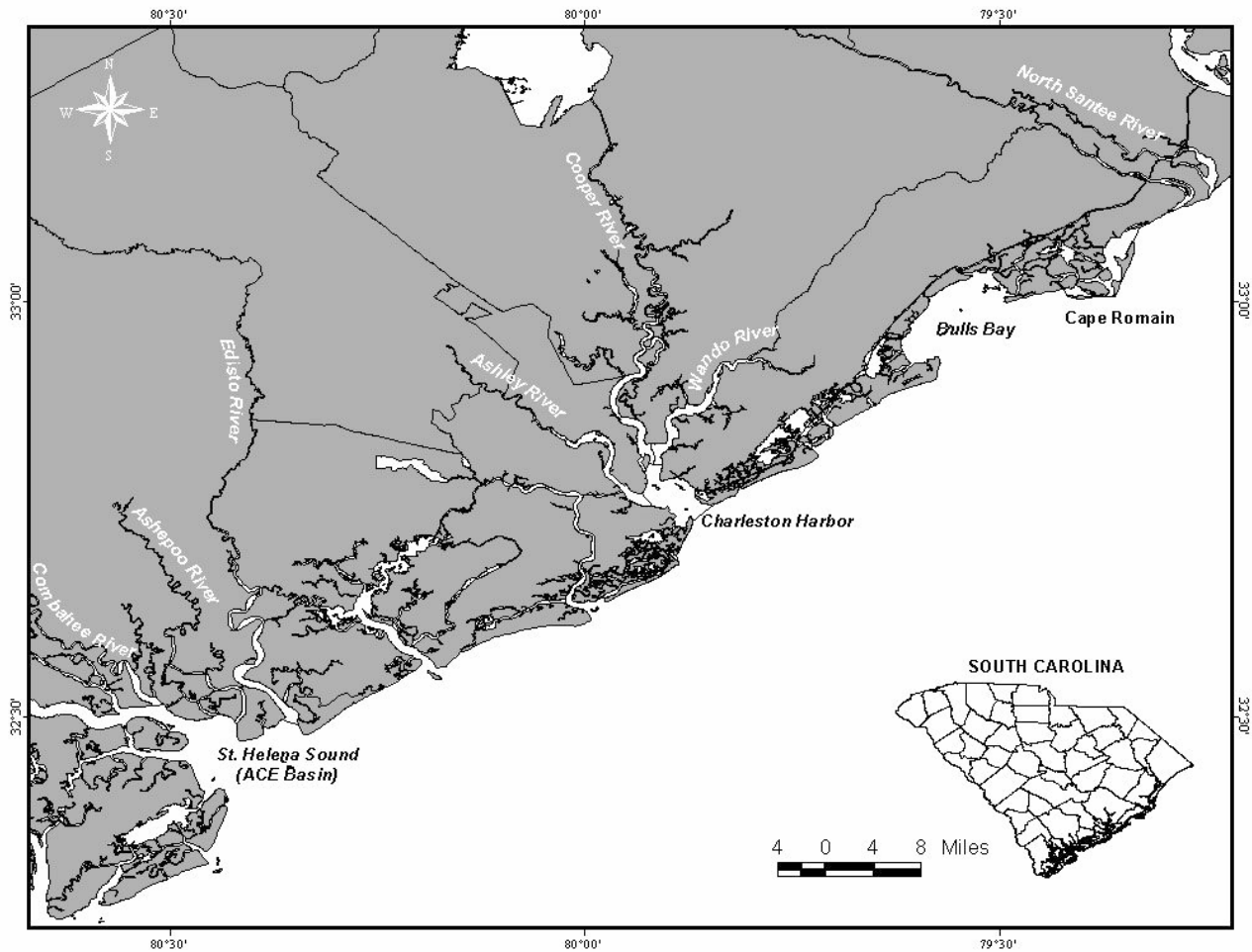
histological baseline for the evaluation and reproductive staging of striped mullet.

## Materials and methods

### Sampling and data collection

Collections of striped mullet were conducted from October 1997 through December 2000. Collections were based on a protocol of monthly random stratified sampling conducted in the Cape Romain, Charleston Harbor, and the ACE Basin estuaries in South Carolina (Fig. 1). The Charleston Harbor estuarine system is made up of three river systems: the Ashley, Cooper, and Wando rivers. In addition, Charleston Harbor proper was sampled as a separate stratum. The ACE Basin estuary is formed by the confluence of the Ashepoo, Combahee, and Edisto rivers and was sampled as a single estuary. One of the problems initially encountered with sampling was the ability to sample striped mullet throughout their estuarine salinity range. The primary sampling gear used was a 184-meter trammel net with 356-mm stretch mesh outside panels and a 64-mm stretch mesh inner panel. Because striped mullet use the full range of estuarine habitats and freshwater, the use of alternate gear was necessary to obtain a representative sample of the population within all salinity regimes. Specimens collected with additional gear types in low salinity and freshwater habitats supplemented those specimens sampled with a trammel net. The additional gear types were an electroshock boat, cast nets, and gill nets. The electroshock boat samples were obtained from the South Carolina Department of Health and Environmental Control from the major coastal river basins in South Carolina, including freshwater portions of the Waccamaw, Black, Pee Dee, Sampit, Santee, Cooper, Edisto, Ashepoo, Combahee, and Broad rivers (Fig. 1). Cast nets were used primarily in different portions of the Charleston Harbor estuary in tidal creeks and in areas where the trammel net could not be used effectively. The cast nets were 1.84 meters in diameter and had 10-mm mesh. The gill net was a 200-meter net with 64-mm stretch mesh that was used to test the efficiency of the trammel net sets.

Standard morphological measurements were total length (TL), fork length (FL), standard length (SL) in mm, and body weight (BW) in grams (g). Any subsequent mention of fish length in the remaining text will be total length unless otherwise noted. Sagittal otoliths were removed for estimating fish ages. A gross examination of the gonads was used for initial sex and maturity assessment. If the gonads were estimated to weigh more than 1 g they were also weighed. A small sample of gonad tissue was removed from the posterior portion of the gonad where the lobes were joined and was fixed in 10% neutral buffered formalin for histological examination. The tissue samples used for histological evaluation were taken from the posterior section of the gonad because earlier developmental stages and differentiation were more evident where the ductwork



**Figure 1**

Map of coastal South Carolina with estuaries where trammel net collections were made: Cape Romain, Charleston Harbor, and Ashepoo River, Combahee River, Edisto River (ACE) Basin, as well as the coastal rivers where electroshock collections were made.

and gonad tissue joined in striped mullet (Chang et al., 1995). Comparisons of oocyte density from different sections of striped mullet ovary have also demonstrated uniform distribution throughout the ovary (Shehede et al., 1973; McDonough et al., 2003). A gonadosomatic index (GSI) was calculated for specimens according to the method of Render et al. (1995) where GSI was expressed as a percentage of gonad weight (GW) divided by body weight (BW) minus gonad weight, such that

$$GSI = (GW / (BW - GW)) \times 100.$$

#### Histological processing

The tissue samples were processed by using standard wax histology techniques (Humason, 1967). Tissues were embedded in paraffin and cut on a rotary microtome. The sections, which ranged from 5 to 7  $\mu$ m thick, were then placed on microscope slides and stained with standard haematoxylin and eosin-Y staining techniques (Humason, 1967). After staining, tissue sections were

sealed under a cover slip and evaluated for sex and maturity with a compound light microscope at 100 $\times$  magnification. The sex of each specimen was determined to be male, female, or undifferentiated. Maturity was assessed according to a modified version of the schedule used by Wenner et al. (1986) that was adapted by the authors to work with isochronal spawning fish, as well as assessed with previous models of reproductive development (Stenger, 1959; Grier, 1981; Wallace and Selman, 1981) (Table 1). Ovarian atresia was divided into four distinct phases as described by Hunter and Macewicz (1985). For the sake of consistency, the same terminology was used to describe the four phases of ovarian atresia in striped mullet in this study: alpha, beta, gamma, and delta (see Table 2). These evaluation methods were based on identification of morphological characteristics evident in histological sections. Specimens were evaluated by two readers to avoid bias. Any discrepancies of maturity stage between readers were either mutually resolved or the specimen was excluded from further analysis.

**Table 1**

Histological criteria used to determine reproductive stage in striped mullet (*Mugil cephalus*) once sexual differentiation has occurred. Modified from Wenner et al. (1986).

Reproductive stage	Male	Female
1. Immature	Inactive testes; small transverse sections compared to those of resting male; spermatogonia and little or no spermatocytic development.	Inactive ovary with previtellogenic oocytes and no evidence of atresia. Oocytes are <80 $\mu\text{m}$ , lamellae lack muscle, and connective tissue bundles are not as elongate as those in mature ovaries, ovary wall is very thin.
2. Developing	Development of cysts containing primary and secondary spermatocytes all the way through accumulation of spermatozoa in lobular lumina and ducts.	Developing ovary have enlarged oocytes generally greater than 120 $\mu\text{m}$ in size. Cortical alveoli become present and actual vitellogenesis occurs after oocytes reach 180 $\mu\text{m}$ in size and continue to increase in size. Abundant yolk globules with oocytes reach a size range of >600 $\mu\text{m}$ ,
3. Running, ripe	Predominance of spermatozoa in lobules and ducts and little occurrence of spermatogenesis.	Completion of yolk coalescence and hydration in most oocytes.
4. Atretic or spent	No spermatogenesis occurring but some residual spermatozoa in shrunken lobules and ducts.	More than 30% of developed oocytes undergoing the atretic process. See Table 2 for detailed description of the atretic process.
5. Inactive or resting	Larger transverse sections compared to those of immature males; little or no spermatocytic development; empty lobules with well-developed secondary ductwork and some residual spermatogonia.	Previtellogenic oocytes with only traces of atresia. In comparison to those of immature females, most oocytes are >80 $\mu\text{m}$ , lamellae have some muscle and connective tissue bundles; lamellae are larger and more elongated than those of immature females and the ovarian wall is thicker.

**Table 2**

Histological criteria used to determine atretic stage in striped mullet (*Mugil cephalus*). Criteria based on ovarian atretic process described by Hunter and Macewicz (1985) and observational data of striped mullet ovaries from this study.

Atretic stage	Description
1. Alpha atresia $\alpha$	Vitellogenic oocytes are present with distinct yolk globules, which are beginning to break down. The most developmentally advanced oocytes will undergo atresia first, followed by less developed oocytes. The oocyte will break down from the interior outward; the vitelline membrane and follicle layers are the last portion of the oocyte to decay. As the oocyte breaks down, a series of vacuoles of various sizes will appear within the oocyte.
2. Beta atresia $\beta$	The oocytes continue to become reduced in size as they decay. The vacuoles that began to form during the alpha stage are now coalescing together to form one large vacuole within the oocyte. This gives the lamellae a distinct hollow matrix and just the outer layers of the oocyte and follicle are now left. This appears to be the shortest atretic phase.
3. Gamma atresia $\gamma$	The oocytes that were left in the hollow matrix during the beta stage now begin to shrink in size and the outer layers fold in on themselves as the oocyte collapses. The areas in and around the collapsed oocytes and lamellae become highly vascularized during this stage in order to facilitate rapid resorption of decaying cellular material. There will still be some vacuoles present within the collapsed oocytes but they have become much smaller and there are far fewer of them. This stage continues until most of the remaining oocytes that developed for spawning are no longer recognizable as oocytes.
4. Delta atresia $\Delta$	The remnants of old oocytes at this stage are identifiable only as decaying cellular material and will stain a distinct yellow-brown color and are still present in (approximately) 30% or more of the material within the ovary. Undeveloped oocytes have a much more distinct and numerous presence within individual lamellae. The amount of vascularization seen in the gamma stage is reduced because most of the old material has been reabsorbed.

## Aging techniques

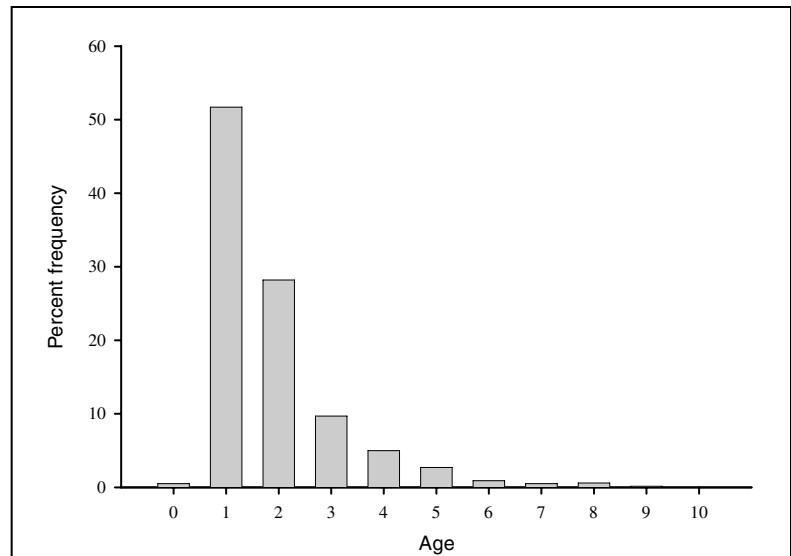
Age was determined by using the left sagittal otolith, which was embedded in epoxy resin. A 0.5-mm transverse section encompassing the otolith core was cut with an Isomet low speed saw with diamond wafering blades. The thin section of otolith embedded in the epoxy was observed with a dissection microscope at 20× magnification, and age was recorded as the number of annular rings present. The otoliths were initially aged by one reader. A second reader then evaluated a subsample of specimens from 1998 and 2000 and all the otoliths from 1999. The two groups of ages were compared by the percentage of agreement between the different age determinations and by a paired *t*-test that allowed a comparison of the means and variances of the two groups (Campana et al., 1995). Ages were then validated by marginal increment analysis in order to establish the timing and periodicity of increment deposition (Campana, 2001). In addition, the precision of the ages was compared by using average percent error (APE) between the two sets of ages. "Precision" was defined as the reproducibility of age determinations (Beamish and Fournier, 1981; Chang, 1982). Using the Levenburg-Marquardt procedure (Zar, 1984), we determined the growth curve with a nonlinear least squares regression of total length on age.

## Results

### Age structure

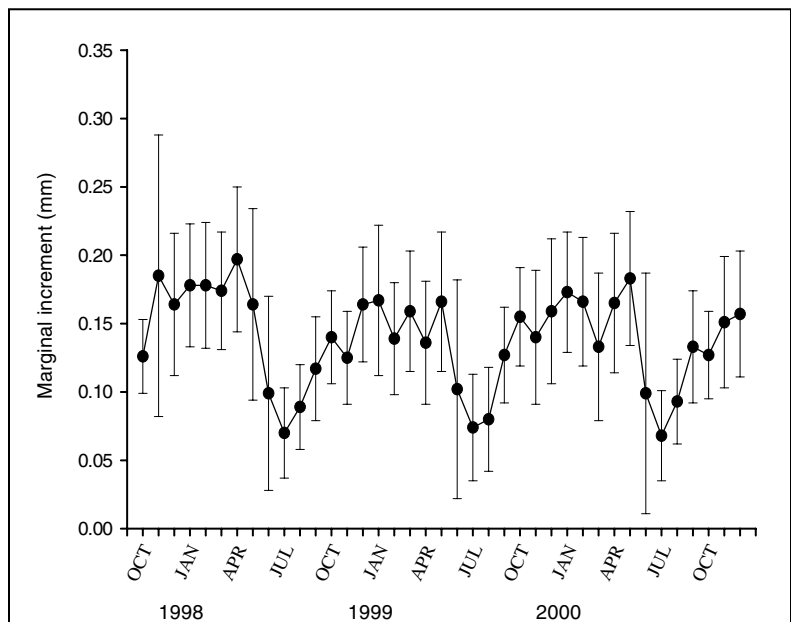
We recorded the age of 3760 specimens and examined these specimens histologically to determine sex and maturity stage. An additional 2524 young-of-the-year (age 0) specimens were used for the nonlinear regression of total length on age, as well as the sex ratios by both size and age. The age range for striped mullet in this study was 0 to 10 years, and 1- and 2-year-olds dominated the age distribution (Fig. 2). There was 81.7% agreement for age data between the two readers, and 99.5% agreement within one year for both readers. A *t*-test indicated no significant difference between the two sets of age estimations ( $t=2.898$ ,  $df=1,233$ ,  $P<0.05$ ). The average percent error (APE) (Beamish and Fournier, 1981) between the two sets of age estimations was 0.41%.

Marginal increment analysis indicated that growth increments were deposited during July (Fig. 3). The



**Figure 2**

Age-frequency distribution (expressed as a percentage) for striped mullet (*Mugil cephalus* L.) from South Carolina estuaries October 1997 to December 2000.  $n=3760$ .



**Figure 3**

Mean marginal increment distance by month for striped mullet (*Mugil cephalus* L.) from South Carolina estuaries, October 1997 to December 2000.  $n=3760$ . Marginal increment equals the otolith section radius minus the distance from the core to the last annular increment.

total length at age regression demonstrated a strong relationship ( $r^2=0.864$ ,  $df=3759$ ,  $F_{stat}=21,742$ ,  $P<0.05$ ). Despite this strong relationship, there was a wide range of sizes among the 1-, 2-, and 3-year-olds (Fig. 4).

There was a lag period between the time of formation of the first annual growth mark and the actual one-year birthdate. The first annular mark was deposited between 15 and 19 months of age or at 1.25 to 1.6 years of age (Wenner and McDonough<sup>3</sup>).

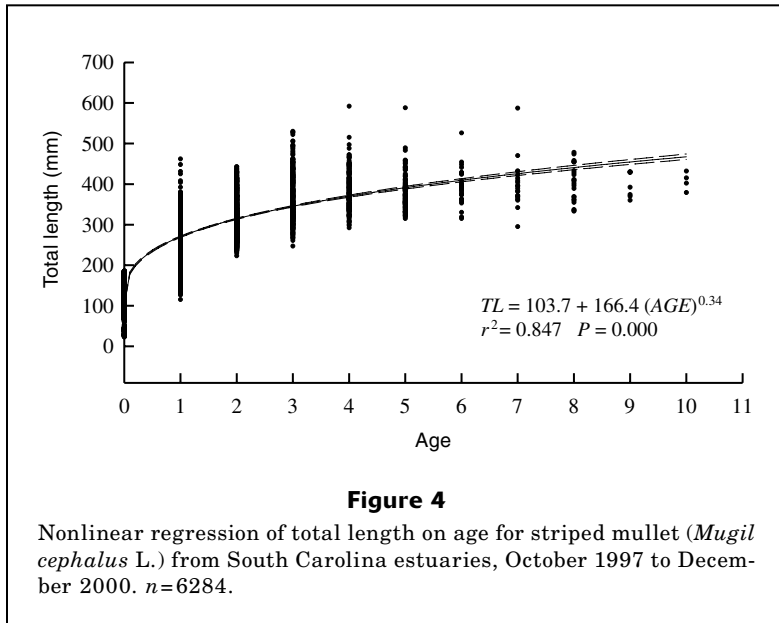
**Sexual differentiation**

The smallest sexually differentiated male was 137 mm (Fig. 5). Male striped mullet 126 to 150 mm TL were eight to twelve months old (McDonough and Wenner, 2003). The first sexually differentiated female was 164 mm TL. Females 151 to 175 mm would have been approximately one year old (McDonough and Wenner, 2003). Specimens greater than 200 mm were at least 50% sexually differentiated. Only 1.5% of specimens over 300 mm remained undifferentiated. The largest sexually undifferentiated specimen was 325 mm. All fish >325 mm, although still possibly sexually immature, were fully sexually differentiated. The ratio of males to females was 2:1 until the fish were larger than 325 mm ( $\chi^2_{\alpha=0.05}=2543.9, df=2$ ). The ratio of males to females was 1:3.8 for fish >325 mm ( $\chi^2_{\alpha=0.05}=352.8, df=1$ ).

The sex ratio by age class showed 98.9% of the age-0 specimens were sexually undifferentiated (Fig. 6). The few age-0 fish that were differentiated were all males. At first annulus deposition, 91.9% of the specimens had differentiated. There were a few specimens (0.8%) that remained undifferentiated to 3 years old, but all striped mullet age 4 or older were completely differentiated. The sex ratio of males to females in the one-year-old age class was 1.0:0.25 ( $\chi^2_{\alpha=0.05}=1065.4, df=2$ ). At age 2 the ratio was 1.0:0.68 ( $\chi^2_{\alpha=0.05}=502.6, df=2$ ) and at age 3 the ratio had reversed to 0.32:1.0 ( $\chi^2_{\alpha=0.05}=312.5, df=2$ ).

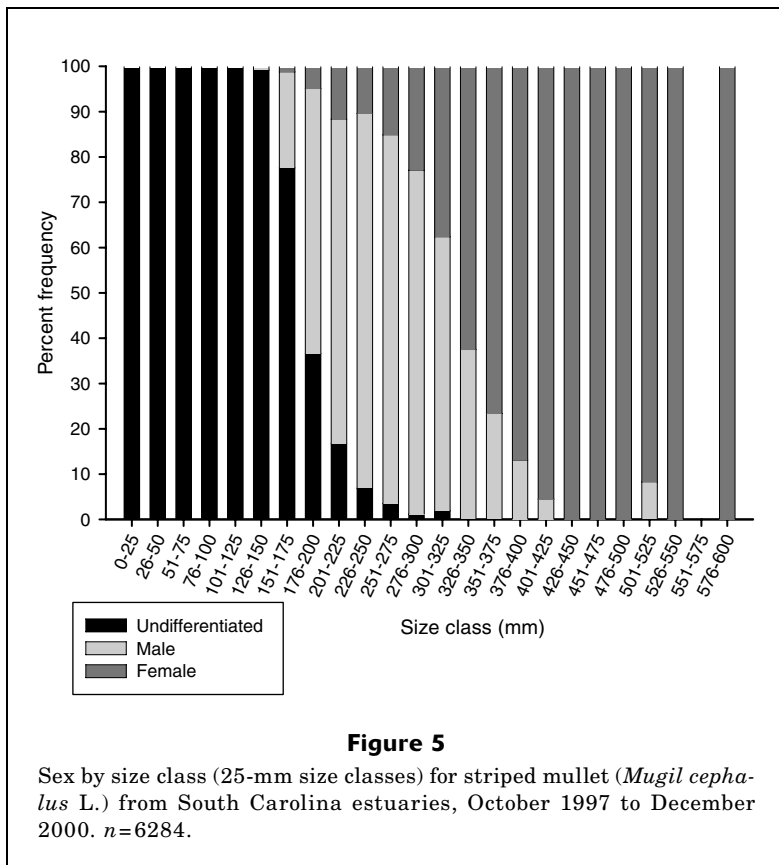
**Size and age at maturity**

The onset of spermatogenesis in males was first observed at 248 mm (Fig. 7A). The first running, ripe males occurred at 291 mm and this developmental stage was found in all larger sizes. Postspawning males were found only between November and March in mullets larger than 325 mm. Resting mature males were found in every month and occurred in most size classes greater than 251 mm. These resting males made up fewer than 50% of the specimens from any particular size class. A small percent-



**Figure 4**

Nonlinear regression of total length on age for striped mullet (*Mugil cephalus* L.) from South Carolina estuaries, October 1997 to December 2000. *n*=6284.



**Figure 5**

Sex by size class (25-mm size classes) for striped mullet (*Mugil cephalus* L.) from South Carolina estuaries, October 1997 to December 2000. *n*=6284.

<sup>3</sup> Wenner, C. A., and C. J. McDonough. 2001. Co-operative research on the biology and assessment of nearshore and estuarine fishes along the southeast coast of the U.S.: Part IV: Striped mullet, *Mugil cephalus*, p. 17-23. Final Report, Grant no. NA77FF0550. Marine Resources Research Institute, South Carolina Dep. Natural Resources, P.O. Box 12559 Charleston, SC 29422-2559.

age (2.5%) of immature males were found in size classes greater than 325 mm. Male striped mullet showed 50% maturity at 275 mm, and 100% maturity by 350 mm.

Oogenesis began in specimens as small as 291 mm (Fig. 7B) and there were 15 females below 325 mm undergoing oogenesis (4.5% of all developing females). Ovaries were found in three small females (<300 mm). Immature females were not found larger than 400 mm or older than 3 years. All females greater than 400 mm were mature, regardless of their age. The majority of females over 425 mm (88.3%) were developing and found only in the fall. No ripe female striped mullet were found. Ovarian atresia was found from December through May. Female striped mullet showed that 50% maturity was reached at 325 mm, and 100% maturity occurred in specimens 400 mm.

Gametogenesis occurred in each sex between the first and second year (Fig. 8). However, the majority of specimens at age 1 (65%) were immature. Males and females showed 50% maturity at 2 years. Males showed 100% maturity at age 4 and females at age 5. Running, ripe males were first observed at age 1 but were found in much greater numbers at ages 4–6. Resting males occurred in every age class except age 6 (Fig. 8A). The abundance of males aged 3 and older was far lower (by at least an order of magnitude) than that of 1- and 2-year-olds (Fig. 8A). Atretic ovaries were found in all age classes, and resting females were found in every age class except age 0 (Fig. 8B).

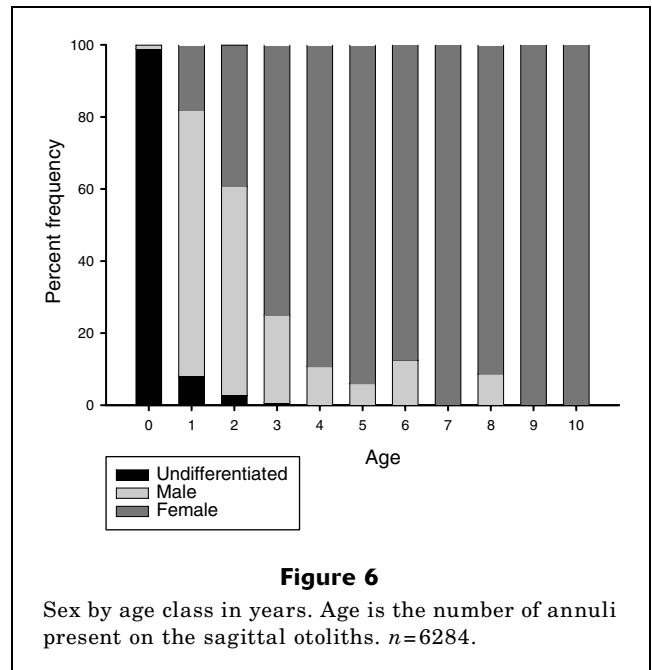
Maturity stages by month showed immature and resting (but sexually mature) male and female striped mullet occurred in every month (Fig. 9). Developing males were found from August through February, and running, ripe males from October through February. Males (atretic) were found from November through March. Developing females occurred from August through April. Mean monthly GSI for males and females showed noticeably increased gonad size in November and December, and obviously enlarged gonads occurred from October through March (Fig. 10).

#### Histological descriptions: undifferentiated juveniles

The primordial gonad lobes were suspended by mesentery connected dorsally to the peritoneum and were attached ventrally to the intestines (Fig. 11A). Gonads from specimens <100 mm were identifiable only through histological examination of whole-body cross sections.

The gonads in specimens less than 50 mm had lobes ranging from 70 to 100  $\mu\text{m}$  in length (Fig. 11B). Lobes were made up of somatic cells and a peripheral germinal epithelium. The lobes were attached along their dorsomedial surface by loose fibrous connective tissue, known as stromal tissue. No defining male or female characteristics were present at this fish length.

In specimens ranging from 50 to 100  $\mu\text{m}$  gonad lobes had increased to 150  $\mu\text{m}$  and appeared more vascularized (Fig. 11C). The lobes were attached to the suspensory mesentery, which was attached to the peritoneum. A few deuterogonia were visible along the lateral pe-



riphery of each lobe. The remainder of the lobes contained somatic tissue. The individual germ cells were approximately 5  $\mu\text{m}$  in size. In some specimens, somatic tissue was beginning to form bands that would later develop into ductwork.

In specimens ranging from 100 to 150 mm, the gonad lobes were obviously vascularized and had attained a size of 200 to 300  $\mu\text{m}$  (Fig. 11D). Early ductwork was beginning to become evident. Deuterogonia were enlarging and forming nests along the lateral and distal portions of the lobes. Somatic cells made up a large portion of each lobe and the stromal tissue was now more stalklike, attaching each lobe to the suspensory tissue. There were only four specimens in this size range that had started to differentiate as males. Gonads destined to be males were identified by duct structures within the gonad lobe as well as by more elongated germ cell nests. These morphologically distinct features resulted in an early demonstration of the corradiating pattern of ducts and lobules seen in more advanced testes.

The 150 to 200 mm size class showed that 0.2% of females and 37.3% of males began initial differentiation, but the majority of all specimens (62.5%) remained undifferentiated. The undifferentiated gonads had become larger, and lobe size was 600 to 800  $\mu\text{m}$  (Fig. 11E). There was increased vascularization, particularly along the medial portion of the stroma. Germ-cell nests were now more organized, with 4 to 8 cells visible in each.

More than 83% of specimens >200 mm had become sexually differentiated. The undifferentiated gonads in specimens >200 mm were highly vascularized and had both the presence of ductwork, rounded germ cell nests, and lobule-like structures. In some cases, germ cell nests that were characteristic of female precursors

could also be found in the center portions of lobes adjacent to the characteristic male precursor lobule structures. The primary duct was now well formed; however there were still no definitive morphological characteristic that would enable sex determination.

### Male differentiation

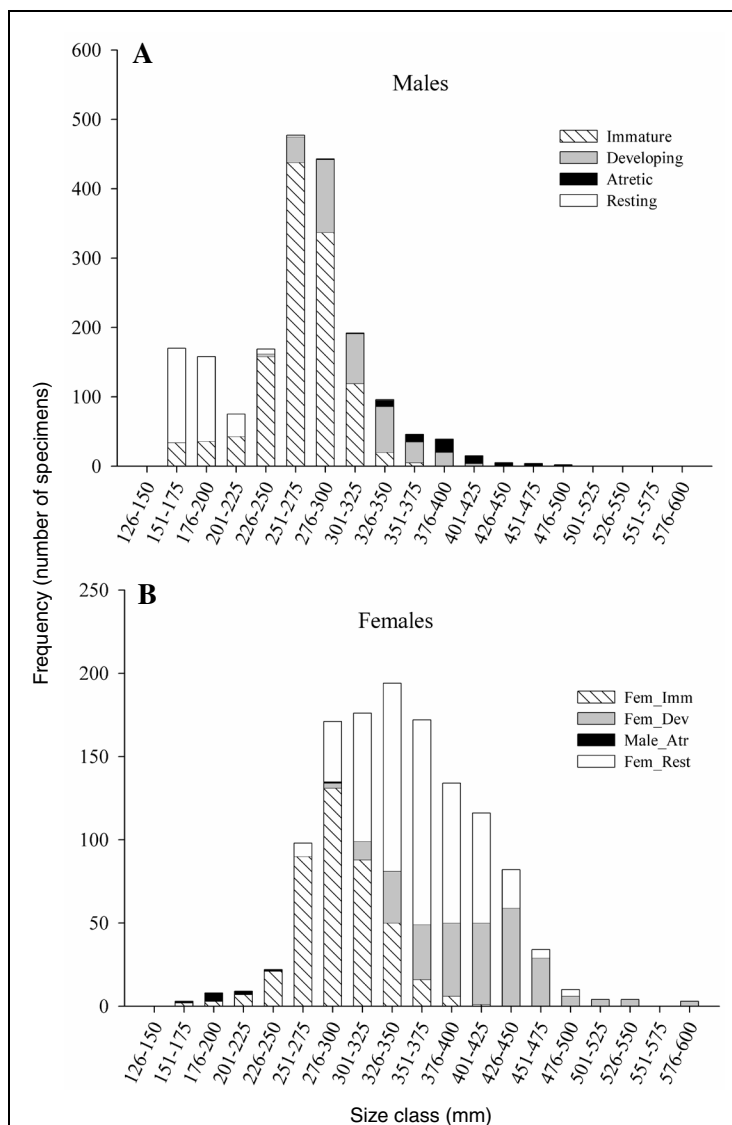
The initial differentiation of males was evident in the morphological features of the germ-cell tissue located along the peripheral portions of each lobe. The germ tissue began to form elongated bands perpendicular to the edge of the lobe, whereas the somatic tissue began to form fibrous bands originating along the edges of the

primary duct (Fig. 12A). The primary duct was defined structurally at this point. With continued increase in fish length, lobes increased in size and vascularization. The germ tissue continued to elongate medially within the lobe in a corradiating pattern (Fig. 12B). Somatic tissue continued to form bandlike structures that would eventually become secondary ductwork, and the germ-cell expanded to form lobules (Fig. 12C). As the lobules became more developed, spermatogonia began to line the lobules as part of the germinal epithelium (Fig. 12D). Sertoli cells were not visible because of the lack of resolution at this magnification (400 $\times$ ) level. Mitotic proliferation of spermatogonia caused lobular enlargement, although spermatogonia were very small at this stage (2–3  $\mu$ m). The overall male aspects of the physical structure of the lobes was clear at this point (Fig. 12E). Melanomacrophages were found in the lobes of some specimens (Fig. 12F). The melanomacrophages were found only in immature and differentiating males.

### Female differentiation

The first sign of female sexual differentiation was the organization of germ-cell tissue into round nests of 8–10 cells each (Fig. 13A). The germ-cell nests, which eventually gave rise to oogonial nests, were first found along the lateral periphery of the lobe and were infrequently scattered within the gonad lobe. There was evidence of early ovarian wall development, which consisted of a single layer of cells forming the outer layer of the lobe, separate from the oogonial nests (Fig. 13B). Although some ductwork was present, there was no evidence of the formation of lamellae. Ductwork tended to become reduced as the germ-cell nests became more numerous. With continued development, individual cells within the nests became more visible and the ovarian wall became more evident (Fig. 13B). Stalks or buds of tissue were observed growing out of the base of the stroma on the dorsolateral surface (Fig. 13C). As development progressed, the ovarian wall attached to these stalks or tissue buds appeared to grow over the dorsal surface of each ovarian lobe. This ovary-wall stalk bud was not necessarily an indicator of female differentiation because a small number of samples (0.6%) with definite male structure also had indications of these stalk buds. However, these tissue stalks were present in 68% of the differentiating females. The presence of both the ovary wall stalk buds and the rounded germ-cell nests located throughout the gonad lobe were diagnostic of female differentiation.

Primary growth oocytes increased in number and began to aggregate, forming distinct lamellae (Fig. 13D). The ovary wall continued to differentiate at this point but was only a few



**Figure 7**

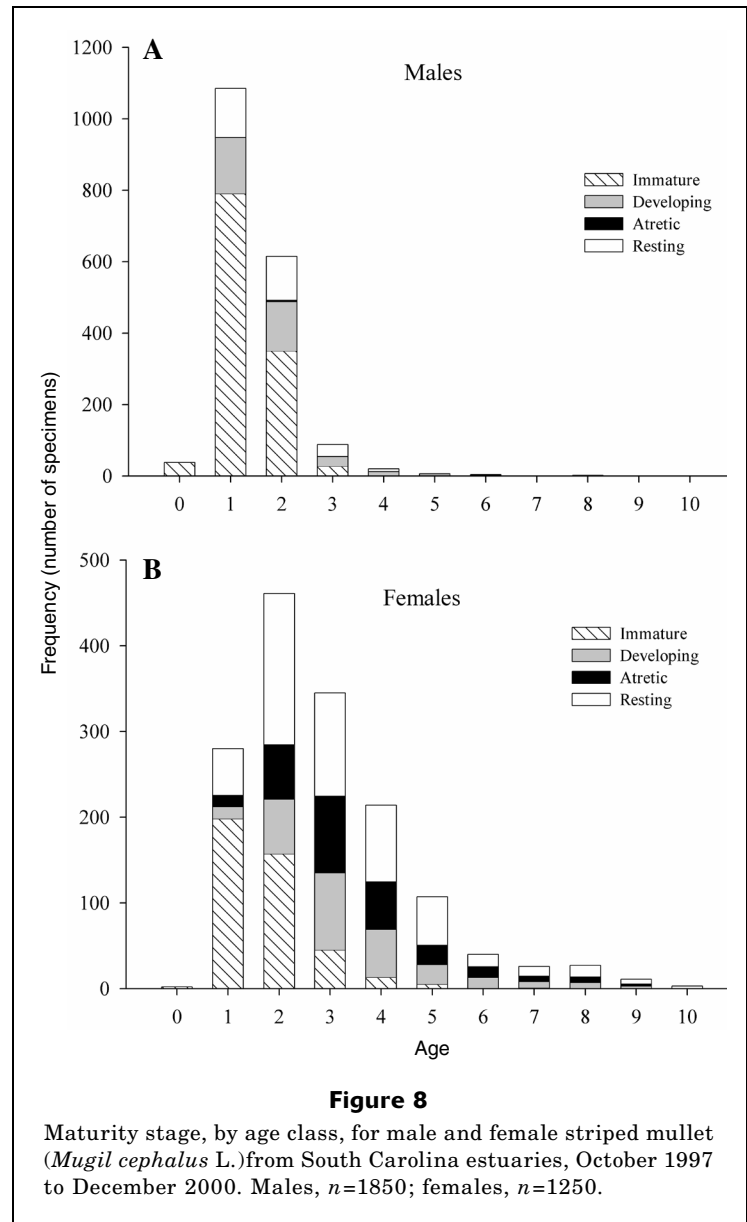
Maturity stage by size class for male and female striped mullet (*Mugil cephalus* L.) from South Carolina estuaries, October 1997 to December 2000. Males,  $n=1850$ ; females,  $n=1250$ .



cell layers thick. There was still a great deal of stroma and somatic tissue left in the ovary, but it began to form bands of fibrous tissue, resulting from the regression of stroma and somatic tissue (where present) as the lamellae continued to develop. The primary duct was greatly reduced. Oogonia began proliferating and differentiating into primary growth oocytes as folliculogenesis commenced. The ovary wall, now becoming vascularized, began to separate from the lamellae, opening a space that would become the ovarian lumen (Fig. 13, D–F). The ovary wall was made up of squamous cells on the inside layers and collagen and elastic tissue on the outer layers. The stroma and somatic cells continued to be reduced until they were primarily fibrous tissue from which the lamellae were suspended. Histological ovarian cross-sections changed from the leaf or spade shape of the undifferentiated gonad to a more rounded one. Once ovarian differentiation was completed, the individual lamellae were seen to have oocytes within each and the stroma was reduced to suspensory tissue for the lamellae (Fig. 13F). The primary growth oocytes present in the lamellae remained small (80 to 100  $\mu\text{m}$ ) and relatively uniform in size. At the initiation of reproductive development, the oocytes started to grow from the arrested prophase of the first meiotic division (Stenger, 1959; Kuo et al., 1974).

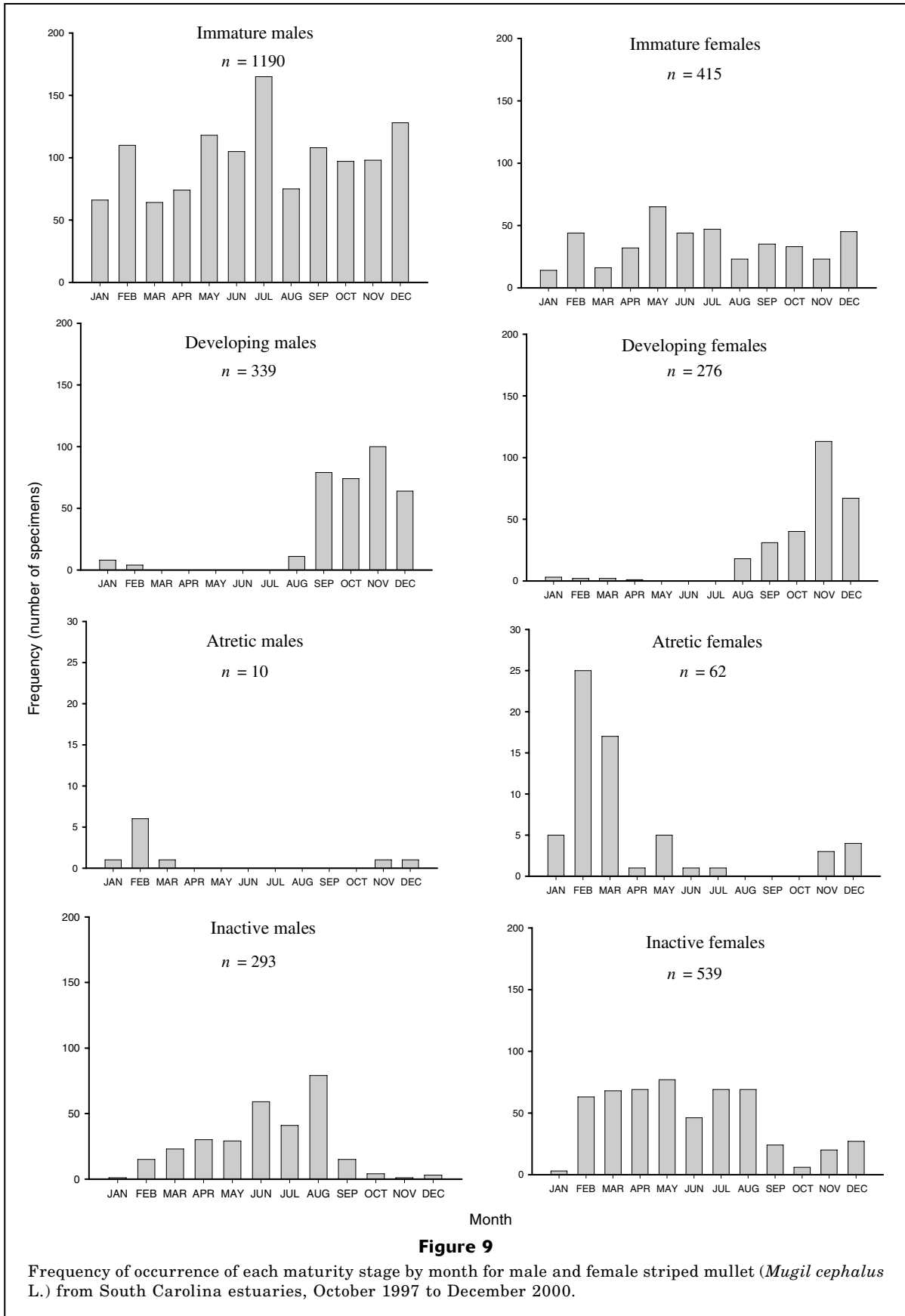
#### Morphological features of atretic females

Females undergoing atresia were captured in all months except August–October, and 78% were seen January–March of each year. The first sign of alpha atresia was the breakdown of the most advanced residual oocytes. Vacuoles (of various sizes) began to appear (Fig. 14A), merging to form large spaces within the decaying oocyte. The overall diameter of oocytes decreased from 600 to 300–400  $\mu\text{m}$ ; however oocytes retained their overall shape during alpha atresia and showed no signs of collapse (Fig. 14B). Beta atresia was the shortest phase. The oocytes had shrunk in size (<300  $\mu\text{m}$ ) but retained their previous overall structure and shape. A distinct hollow matrix retaining only the outer layers of the oocyte (follicle layers and the vitelline membrane) was the defining characteristic for beta atresia (Fig. 14C). The tissue retained this structure while the oocyte continued to decrease (150–180  $\mu\text{m}$ ). During gamma atresia the oocytes collapsed (Fig. 14D) or shrank. Some vacuoles remained in partially collapsed oocytes, but they were fewer in number and smaller in size (<150  $\mu\text{m}$ ) (Fig. 14E). The areas in and around the collapsed oocytes and ovarian lumen became more vascularized during this stage, and this helped facilitate rapid resorption of decaying cellular material (Fig. 14F). Undeveloped oocytes became more visible and numerous. Gamma atresia ended when only masses of broken-down



cellular material remained. Delta atresia was characterized by the presence and decay of nondescript cellular material from the previous spawning (Fig. 14G). Delta atresia was present in approximately 30% or more of the ovary. There was also a decrease in the amount of vascularization within the ovarian lamellae during this stage because most of the old oocyte material had been resorbed. The lamellae contained only undeveloped oocytes and all the remaining material from the previous spawn was concentrated medially in the lamellae.

In the resting stage, no reproductive activity occurred in the ovaries. Infrequently, resting ovaries showed some minor evidence of the previous spawning. The remaining undeveloped oocytes were previtellogenic and varied widely in size (80–120  $\mu\text{m}$ ). The ovary wall was relatively thick, particularly in comparison to the



immature ovaries, and had distinct smooth muscle layers (Fig. 14H). Any stromal tissue left in the ovary at this point was also greatly reduced and was essentially the mesentery from which the lamellae were suspended.

## Discussion

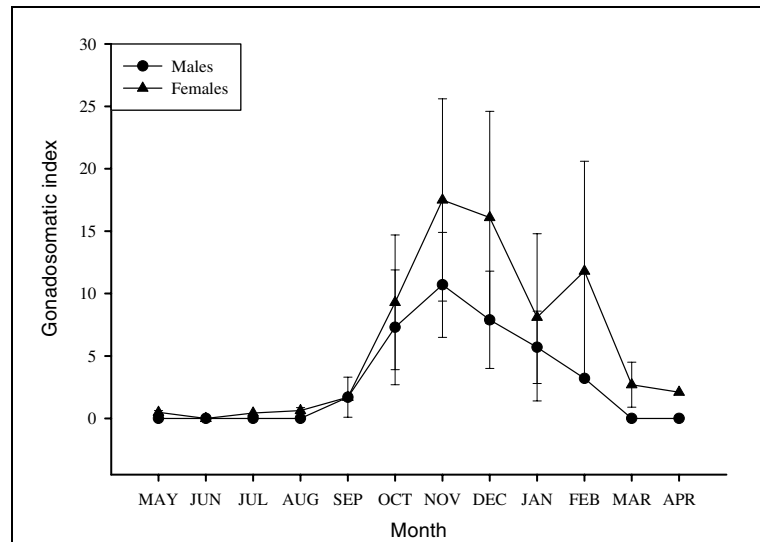
### Age structure

The abundance of 1- and 2-year-old striped mullet in South Carolina indicated that immature fish dominate the estuarine population. The importance of proper age validation in order to make comparisons of age and sexual maturity cannot be understated. The most important aspect of age validation is to obtain a degree of precision that allows repeatability in age determinations (Campana, 2001). The periodicity of growth increment formation was validated by marginal increment analysis, and the precision of these age estimates was then tested by comparing age counts of two independent readers.

Marginal increment analysis showed that annual growth increments were deposited in striped mullet in July in the entire data set, as well as separately for ages 1–5. By validating increment periodicity separately for different age groups, a consistent pattern for the species can be determined (Campana et al., 1995; Campana, 2001). The percent agreement between the two readers and a *t*-test for independent age determinations allowed direct comparisons of the two groups of ages for consistency (Campana et al. 1995). However, these two methods were both independent of the age of the species. Therefore, average percent error (APE) was used to compare the different sets of ages because it is not independent of the age of a species (Beamish and Fournier, 1981). The low APE (0.41%) found between the two different age estimates indicated a high degree of precision, which allowed acceptance of these age determinations.

### Sexual differentiation

Striped mullet are gonochoristic and sex is genetically determined. In contrast to mammals, gender of the mature germ cells of teleosts present in the gonad rather than the gender of the duct system forms the basis for classifying an individual as male or female (Shapiro, 1992). Early duct structures of the undifferentiated gonad characteristic of male development regress on female development. Initial duct development, along with germ tissue placement, takes on characteristics of the eventual sex once the process of differentiation begins (Asoh and Shapiro, 1997). Because of the plasticity of their gonad development, striped mullet retain some characteristics of the opposite sex (such as singular oogonia in males or



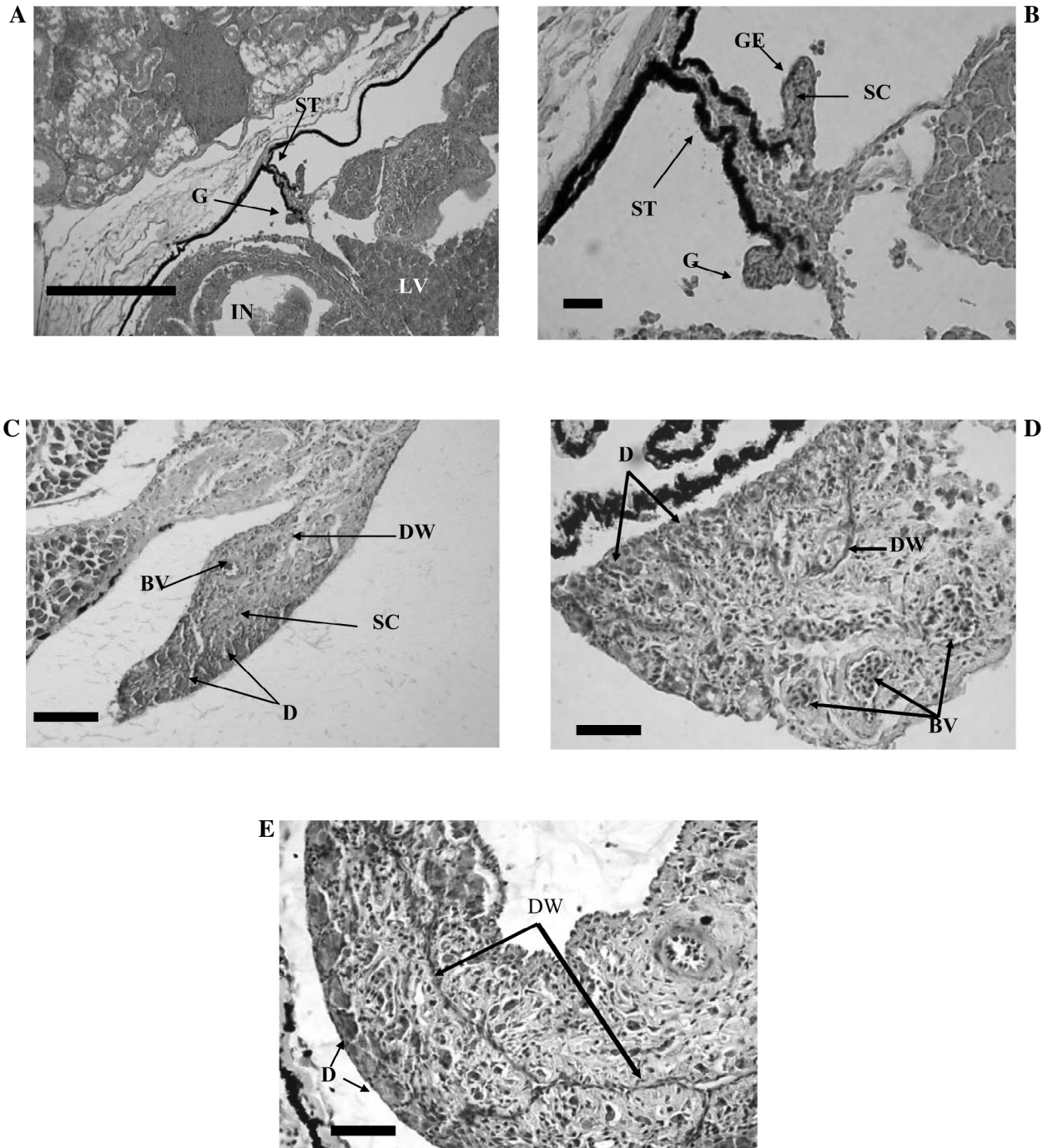
**Figure 10**

Mean gonadosomatic index value by month for male and female striped mullet (*Mugil cephalus* L.) from South Carolina estuaries from 1998 to 2000.  $n=455$ .

duct-work in females) during the initial stages of differentiation. The term that has been used to describe this phenomenon is “intersex” (Yamamoto, 1969) but this state could more accurately be defined as the hermaphroditic stage of some gonochoristic species. Numerous descriptions of intersex exist for teleosts (Atz, 1964). Previous studies have brought up the possibility of hermaphroditism in striped mullet (Stenger, 1959; Atz, 1964; Moe, 1966); however, there is only one example of a simultaneous hermaphroditic striped mullet in the literature (Franks et al., 1998). Once differentiation advances, these secondary characteristics atrophy, and the gonad develops toward the genetically determined sex.

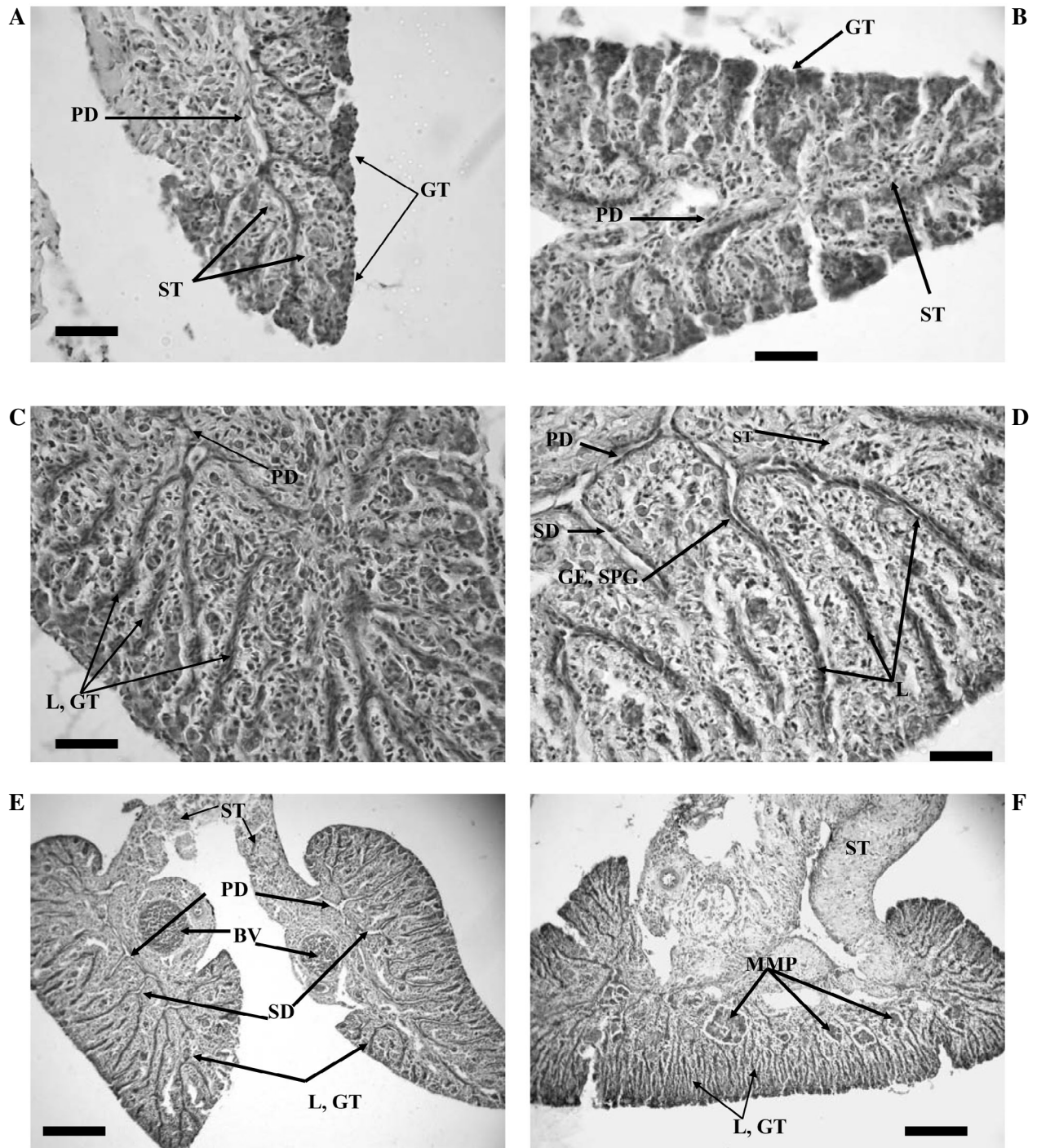
We found that at first annular increment deposition (15–19 months), most (95%) immature striped mullet were sexually differentiated. Chang et al. (1995), using cultured striped mullet, found that differentiation began only after 12 months of age, and 70% to 90% of immature fish at 15 to 17 months had differentiated sexually. We found only a small percentage (1.2%) of differentiated specimens at 12 months of age. Chang et al. (1995) did not report fish sizes, and Stenger (1959) studied sizes at sexual differentiation without reporting age. Stenger (1959) concluded that striped mullet up to 150 mm generally were not differentiated sexually. We found four specimens in which differentiation had occurred in the 126–150 mm size range, which represented specimens 12 months or less in age. Once our specimens reached the 176–200 mm size range, just over 60% had sexually differentiated, which was also approximately the size range at which the first annulus appeared (Wenner and McDonough<sup>3</sup>).

Chang et al. (1995) found that females differentiated earlier than males; we, on the other hand, showed sex



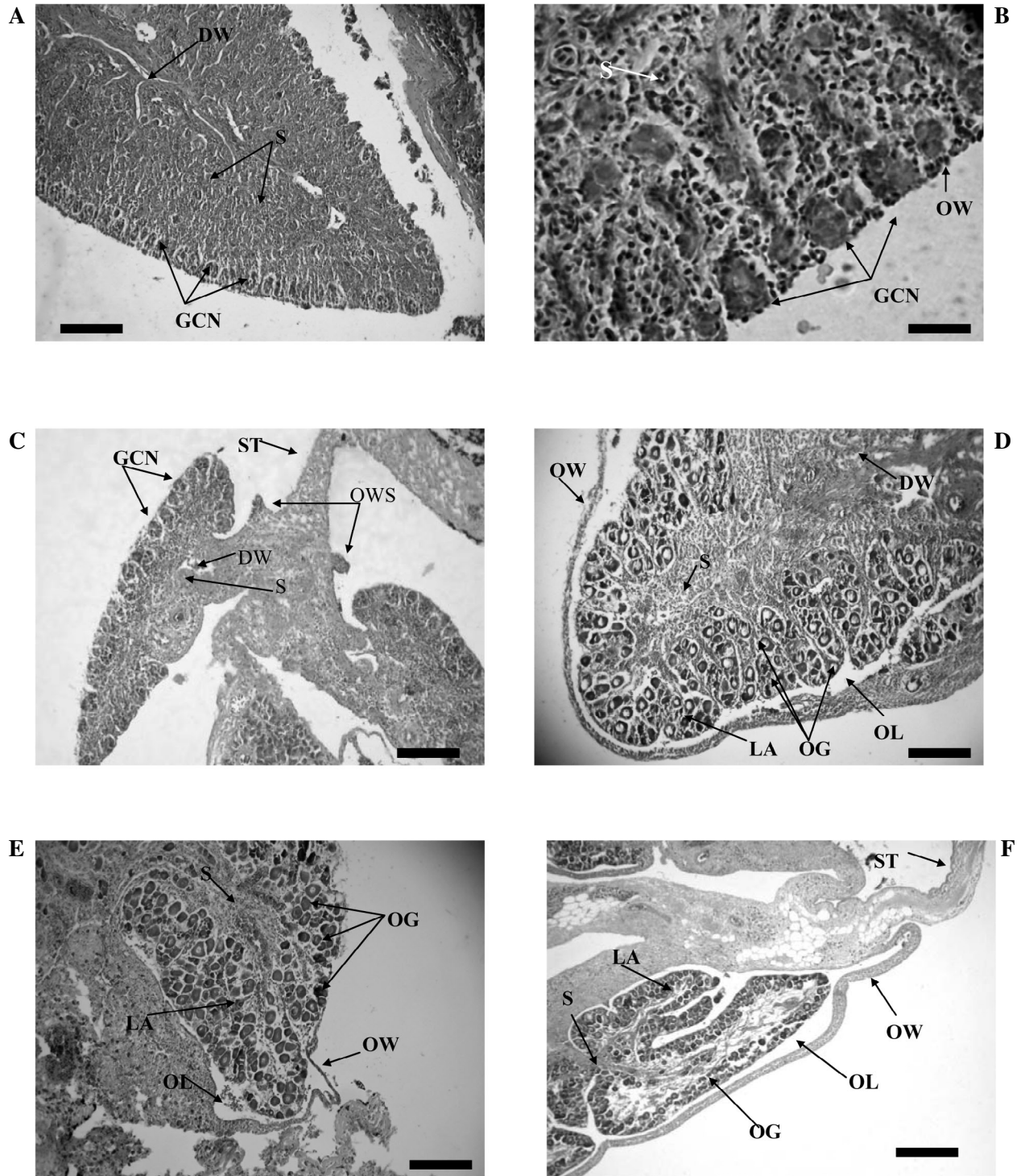
**Figure 11**

Photomicrographs of histological sections of undifferentiated juvenile striped mullet (*Mugil cephalus* L.) (A) 35-mm specimen at 100 $\times$  (scale bar=50  $\mu$ m) and (B) 600 $\times$  (scale bar=10  $\mu$ m) respectively; (C) 55-mm specimen at 400 $\times$ , scale bar=20  $\mu$ m; (D) 135-mm specimen at 400 $\times$ , scale bar=20  $\mu$ m; (E) 184-mm specimen at 400 $\times$ , scale bar=20  $\mu$ m (see text for detailed descriptions of each). Labels: G = primordial gonad; GE = germinal epithelium; SC = somatic cells; D = deuterogonia, DW = duct work; BV = blood vessel; ST = suspensory tissue; LV = liver; IN = intestine.



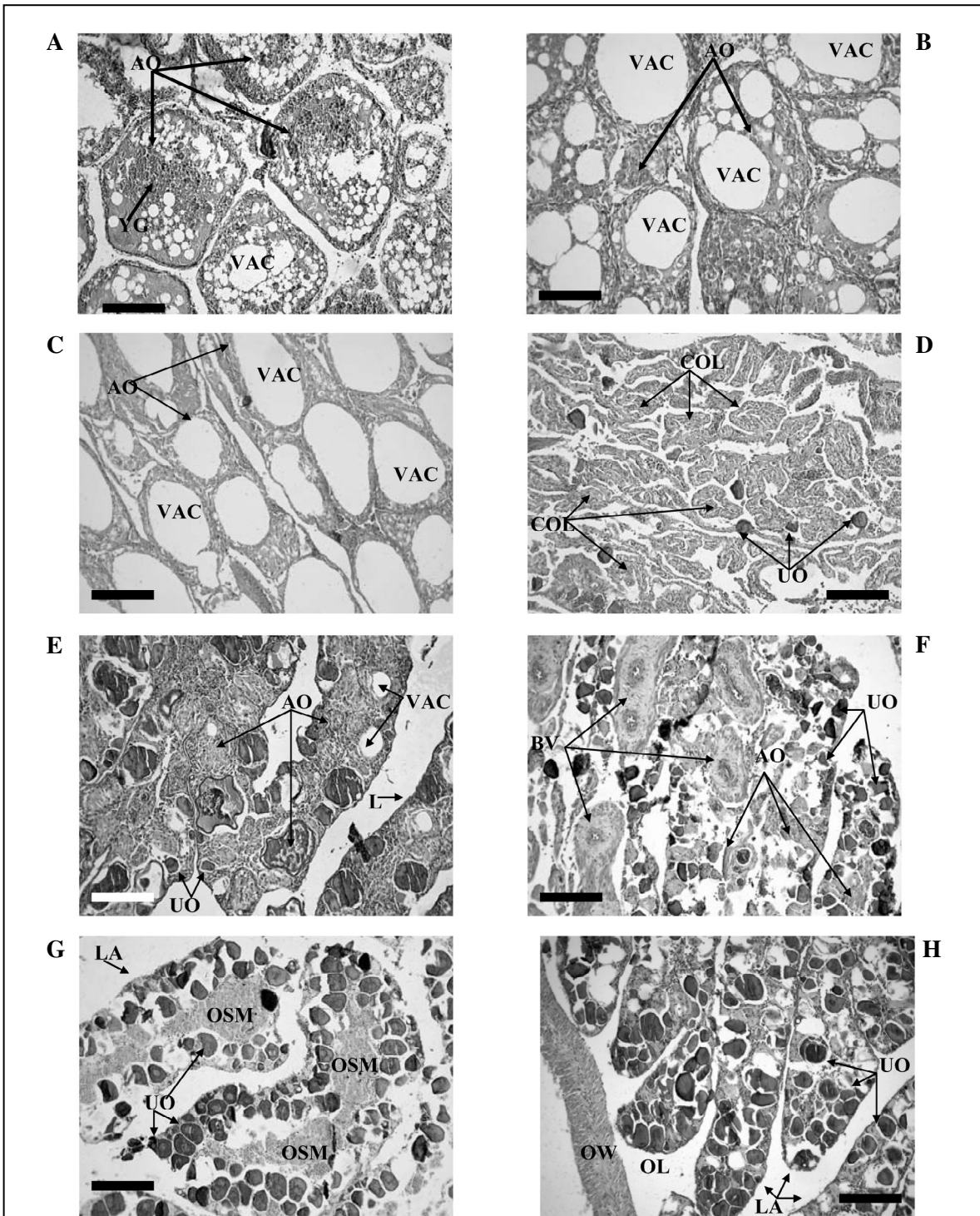
**Figure 12**

Photomicrographs of histological sections of sexually differentiating male striped mullet (*Mugil cephalus* L.). (A) Early differentiation of a 164-mm specimen at 400 $\times$ , scale bar=20  $\mu$ m; (B) early differentiation of a 204-mm specimen at 400 $\times$ , scale bar=20  $\mu$ m; (C) advanced sexual differentiation of a 247-mm specimen at 100 $\times$ , scale bar=100  $\mu$ m; (D) advanced sexual differentiation of a 258-mm specimen at 400 $\times$ , scale bar=20  $\mu$ m; (E) same specimen as previous photo at 100 $\times$  showing full differentiation, scale bar=250  $\mu$ m; (F) differentiated testes with melanomacrophage centers present in a 261-mm specimen, scale bar=250  $\mu$ m (see text for detailed descriptions). Labels: ST = somatic tissue; GT = germ tissue; PD = primary duct; SD = secondary ductwork; BV = red blood vessels; GE = germinal epithelium; SPG = spermatogonia; MMP = melanomacrophages; L = lobule.



**Figure 13**

Photomicrographs of histological sections of sexually differentiating female striped mullet (*Mugil cephalus* L.). (A) Early differentiation of germ cell nests in a 239-mm specimen at 100 $\times$ , scale bar=100  $\mu$ m; (B) early differentiation of germ cell nests in a 205-mm female at 400 $\times$ , scale bar=20  $\mu$ m; (C) mid-differentiation in a 225-mm female at 100 $\times$ , scale bar=100  $\mu$ m; (D) advanced sexual differentiation with developing lamellae and ovarian wall in a 279-mm female at 100 $\times$ , scale bars=100  $\mu$ m; (E) advanced sexual differentiation in a 267-mm female at 100 $\times$ , scale bar=100  $\mu$ m; (F) full differentiation of a 284-mm female, scale bar = 100  $\mu$ m. Labels: GCN = germ cell nests; OW = ovary wall; ST = suspensory tissue; OWS = ovary wall stalks; S = stroma; OG = oogonia; LA = lamellae; DW = ducts; OL = ovarian lumen.



**Figure 14**

Photomicrographs of histological sections of ovarian atresia and the inactive reproductive stage in mature female striped mullet (*Mugil cephalus* L.). (A) Alpha-stage oocyte atresia, scale bar=100  $\mu$ m; (B) Late alpha-stage oocyte atresia, scale bar=100  $\mu$ m; (C) Beta-stage oocyte atresia, scale bar=100  $\mu$ m; (D) Early gamma-stage oocyte atresia, scale bar=100  $\mu$ m; (E) Gamma-stage oocyte atresia, scale bar=100  $\mu$ m; (F) Late gamma-stage and early delta-stage oocyte atresia, scale bar=100  $\mu$ m; (G) Delta-stage oocyte atresia, scale bar=100  $\mu$ m; (H) Reproductively inactive striped mullet ovary with degraded cellular material from previous spawning, scale bar=100  $\mu$ m. Labels: YG = yolk globules; VAC = vacuoles; AO = atretic oocytes; COL = collapsed outer cell layers; UO = undeveloped oocytes; BV = blood vessels; OSM = old spawn material; OW = ovary wall; OL = ovarian lumen; LA = lamellae.

ratios at size indicating that males differentiated first. This difference may be explained by the experimental methods because the differentiation process was likely similar between the cultured and wild fish. Chang et al. (1995) showed that female development occurred before male development based on levels of plasma sex steroids. However, this finding was later corrected to show that plasma sex steroid levels were the same for males and females throughout sexual differentiation (Chang et al., 1999).

The formation of lobules with the proliferation of germ tissue has been previously described as a male developmental pattern (Stenger, 1959; Grier, 1981; Grier and Taylor, 1998; Grier, 2000). The morphological progression seen in the present study was similar to that previously described in histological examinations of differentiation in striped mullet in conjunction with size (Stenger, 1959) and age (Chang et al., 1995). However, ours is the first study to examine sexual differentiation of both male and female striped mullet with changes in size and age and to describe these changes histologically.

The undifferentiated gonad appeared to have male morphological characteristics. The first morphological signs of female differentiation were the movement of deuterogonial germ-cell nests from the periphery of the gonad. This pattern of development was similar to the ontogeny of differentiation described previously for striped mullet (Stenger, 1959). However, the presence of the tissue stalk at the base of the suspensory tissue, to which the ovary wall was attached, has not previously been described. The tissue stalk was present on the majority (68%) of differentiating ovaries and only a few (0.25%) of the differentiating testes. The presence of this stalk in differentiating testes indicated that this characteristic alone should not be used to determine genetic sex. However, the presence of the tissue stalk, in addition to the rounded oogonial nests throughout the gonad, strongly indicated that the specimen was female. There were no specimens observed to be developing an ovary wall that also had developing lobules or duct-work (male characters). Therefore, from a morphological standpoint, the initial definitive identification of the differentiating ovary was the formation of the ovary wall along with rounded germ cell nests throughout the lobe. A primary duct at the center of the developing ovary was present at this stage but any secondary duct-work had begun to atrophy. It was also observed that oogonial and oocyte proliferation could occur throughout the lobe without a definitive ovary wall, which would also be a strong indicator of the female sex.

#### Size and age at maturity

Once sexual differentiation had occurred, the earliest indication of spermatogenesis occurred at just under 250 mm (two specimens) and one year of age. However, the majority of the developing specimens (89.9%) did not show signs of spermatogenesis until they reached

300 mm and age 2. The greater abundance of immature males under 300 mm would also indicate that full maturity was reached by this length. Almost all the males over 325 mm were in some state of reproductive activity, either developing or running, ripe, because most of these larger males were captured only during the spawning season. October, November, and December were the only months when we saw these larger fish, except for some atretic-stage specimens taken from freshwater during the spring. The first signs of spermatogenesis for striped mullet, both from eastern Florida (Stenger, 1959) and South Carolina, were found in August.

Greeley et al. (1987) did not age female striped mullet but used the growth schedule of Thomson (1966) to conclude that striped mullet in eastern Florida reach sexual maturity at 2.25 to 2.5 years, which is 1 to 2 years earlier than that previously reported (Jacot, 1920; Broadhead, 1956; Anderson, 1958; Thomson, 1966). One problem in earlier studies was the use of scale-based age estimates (Jacot, 1920; Thomson, 1951, 1966; Timoshek, 1973). The otoliths used in our study showed more repeatability than would scales. Age schedules based on scales were likely to contain problems with the error terms and overestimation. Another factor may have been the lack of a proper age-validation protocol. The lag in time between the actual birthdate and the first increment formation was not incorporated into the age model. A fish with a single annular ring that appeared to be mature could actually have been up to 30 months old. Male striped mullet did not begin to mature until they were one year old, and almost 100% had reached sexual maturity by age 3. Ripe and atretic males were also found at age 1.

Size at maturity for female striped mullet was reported to be from 290 to 430 mm (Thomson, 1951, 1966; Broadhead, 1956; Chubb et al., 1981). This wide range in size at maturity depended on whether gonads were examined by gross morphological examination (Thomson, 1951, 1966; Broadhead, 1956) or histologically (Stenger, 1959; Chubb et al., 1981). Stenger (1959) found that oocyte development occurred in specimens as small as 270 mm fork length (300 mm TL). Greeley et al. (1987) reported the minimum size at maturity for female striped mullet in eastern Florida was 230 mm SL (290 mm TL). The minimum size at which a female was found to be undergoing active vitellogenesis in the present study was 291 mm. The first signs of female maturity were evident in small numbers (15) in the 2-year-olds. The first atretic females were also found at age 2. The age at maturity for female striped mullet in our study was similar to that found by Greeley et al. (1987) who used length-based predicted ages. Therefore, it appears that striped mullet in South Carolina have a similar maturity schedule to those found in eastern Florida.

Immature and inactive males and females were found every month of the year. The presence of ripe males from October through February and the presence of developing females from August through March support the idea of an extended spawning season from October through April.



The presence of developing females indicated reproductive activity through April: however numbers were small (McDonough et al., 2003). Most of the specimens collected in March and April were either immature or inactive. It has also been demonstrated that striped mullet in closed freshwater systems, such as impoundments, can begin reproductive development. However, unless artificially manipulated, spawning did not occur in freshwater and the fish resorbed the developed gametes (Shireman, 1975; Tamaru et al., 1994). The re-absorption of gametes would undoubtedly have a positive effect on growth rates and may contribute to some of the variation in size at age. Reproductively inactive (but mature) females present every month could indicate that mature mullet do not spawn every year or that fish that remain in the estuary do not migrate offshore to spawn. The most likely possibility would be that inactive females found in the early part of the spawning season may not spawn until much later. However, the presence of developing oocytes beginning in August would indicate that a few months were required for complete recrudescence. It has been shown that striped mullet undergoing the spawning migration between the Black Sea and the Sea of Azov required two months for full ovarian development (Apekin and Vilenskaya, 1978). Also, inactive females from the mid to late spawning season could have spawned early, returned to the estuary, and their ovaries could have regressed. We found no ripe female mullet in the estuaries during the entire study; their absence was likely due to their migration from coastal waters. Evidence of striped mullet spawning (through the back calculation of birthdates from daily growth increments from juveniles) has also shown that the spawning season extends from October through April (McDonough and Wenner, 2003).

### Sexual development

It is not known what cue initiates gametogenesis in striped mullet, but it is generally accepted that changes in temperature and photoperiod help regulate the seasonal reproductive cycle (Anderson, 1958; Kuo et al., 1973; Greeley et al., 1987; Kelly et al., 1991; Render et al., 1995). It has been shown that although striped mullet can mature in a range of salinities, the best production is reached when their gonads develop in salinities of 13 to 35 ppt (Brusle, 1981; Tamaru et al., 1994). Previous studies of striped mullet (Kuo et al., 1974) and other fall spawning fishes that migrate offshore to spawn (de Vlaming, 1974; McQuarrie et al., 1978; Whitehead et al., 1978) have indicated that a shortening day length was the key stimulus for annual reproductive development and migration. Dindo and MacGregor (1981) demonstrated a high correlation between the levels of serum gonadal steroids and the gonadosomatic index in striped mullet during the reproductive cycle; a shortening photoperiod was suggested as the major factor in stimulating reproductive activity. In our study the most reproductively advanced specimens (late recr-

descence) in freshwater were captured in October and no other specimens of similar development were captured during the rest of the spawning season in freshwater. In contrast, the majority of the specimens undergoing vitellogenesis were captured in the lower portions of the estuaries during November and December in salinities greater than 15 ppt. This finding indicated movement of these developing fish from the freshwater portions of the estuary toward the ocean for the spawning migration. This migration time-period also coincided with a mean monthly temperature decrease in temperature (from 21.8° to 13.6°C) and in photoperiod in both the freshwater and brackish portions of the estuaries.

The ovarian atretic process in female striped mullet was characterized by four distinct stages that followed a very similar progression to that described for the northern anchovy (Hunter and Macewicz, 1985). Our study is the first to describe the atretic process in striped mullet ovaries in detail and to apply the classification system developed by Hunter and Macewicz (1985). Knowledge of ovarian atresia is useful for the timing of spawning. However, the lack of immediate atretic-stage fish, with indicators such as postovulatory follicles, prevented us from determining the temporal duration of the different atretic stages. The detailed morphological descriptions of ovarian atresia presented in our study would be of value for future studies to determine the specific timing of the atretic process.

The histological descriptions for male and female developmental stages in association with both size and age data provide a clear picture of these parameters at differentiation and maturity in South Carolina striped mullet. Previous studies of striped mullet reproduction concentrated on just one sex or used cultured fish extensively and may have considered size or age but not both in a single study. Because of the length of the undifferentiated gonad stage in juvenile striped mullet, previous studies have proposed the possibility of protandric hermaphroditism in this species. However, the results of our study indicated that striped mullet are gonochoristic but capable of nonfunctional hermaphroditic characteristics in differentiated mature gonads. It is hoped that the descriptions of developmental morphological features presented in the present study will be useful for future studies by providing a key to reproductive ontogeny that relates directly to somatic growth and age in striped mullet. In particular, the morphological characteristics of sexual differentiation could enable more precise determinations of sex in immature mullet, which, in turn, would indicate the sex ratio of males and females in a given population and allow the development of better management strategies.

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