Maturity, sexual transition, and spawning seasonality in the protogynous red grouper on the West Florida Shelf

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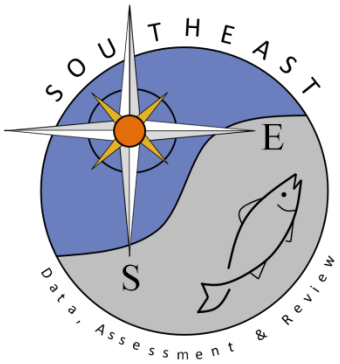

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Maturity, sexual transition, and spawning seasonality in the protogynous red grouper on the West Florida Shelf

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Introduction:

Red grouper, *Epinephelus morio*, support important commercial and recreational fisheries from the southeastern United States Atlantic Ocean, the Gulf of Mexico, and the Caribbean. The Gulf of Mexico (GOM) and South Atlantic fishes are considered separate stocks with a boundary off of the Florida Keys. In the GOM, red grouper support an important commercial fishery, making up roughly two thirds of the shallow water grouper commercial fishery. The center of this fishery is on the west Florida shelf in the northeastern Gulf (Schirripa et al., 1999).

Although the stock is currently not considered 'overfished' or undergoing 'overfishing'(SEDAR, 2009), questions remain on the underlying population structure and potential spatio-temporal effects on maturation schedules and size/age at transition. Because red grouper are protogynous hermparhodites these parameters and the underlying spatio-temporal drivers and density-dependent feedback loops can have important implications for long-term productivity (Alonzo and Mangel, 2004, Brooks et al., 2008, Shepherd et al., 2013), potentially leading to a depletion of males that could result in the Allee effect (Walker et al., 2010).

Red grouper are a moderately long-lived species with a maximum age in the Gulf of Mexico of 29 years. Female maturity (50% mature) is estimated at 3 years old, with 50% males at 11 years old. The objectives of this work were to: (1) develop histological indicators to assess maturity, reproductive timing, transition, and reproductive phases (Lowerre-Barbieri et al., 2011a, Brown-Peterson et al., 2011); and (2) based on these indicators evaluate maturity, size and age at transition, and potential spatial effects.

Survey Design, Sampling Methods, and Analyses:

Reproductive samples of red grouper were collected in association with several fishery-independent surveys and short-term studies conducted by FWC from 2008 – 2013. Red grouper were collected from two trawl surveys: a baitfish survey conducted in April and May in the coastal waters (< 30 m) off Tampa Bay and Charlotte Harbor and the SEAMAP survey conducted in summer (June – July) and fall (October – November) in depths from 10 – 110 m throughout the eastern Gulf of Mexico (Table 1; Figure 1). Both surveys were conducted annually from 2008 – 2013. In terms of traps, most red grouper were collected in chevron traps deployed in association with annual summer (June – September) monitoring efforts (2008 – 2013) off of Tampa Bay and Charlotte Harbor (26° N to 28° N latitude) in depths from 10 – 110 m. Additional samples were collected in association with special trapping projects conducted in the Florida Middle Grounds (2008 and 2009), the Dry Tortugas (2008 – 2011), and the Elbow Reef Area off of Tampa Bay (2012 and 2013); most red grouper were collected using chevron traps, although exploratory Z traps were also deployed in association with the Elbow Reef Area study. Red grouper were also collected in association with several exploratory hooked-gear surveys. These studies used a variety of different hooked gears, including vertical longlines, short bottom longlines, and active hook-and-line fishing, although terminal tackle was generally consistent among studies (e.g., 8/0, 11/0, and 15/0 circle hooks). Special hooked-gear studies were conducted in the Florida Middle Grounds (2008 and 2009), the Dry Tortugas (2008 – 2011), off of the Florida Panhandle and Tampa Bay/Charlotte Harbor (2009 – 2011 and 2013), and the Elbow Reef Area (2012 and 2013). With the exception of the 2009 – 2011 study, which was conducted quarterly, most hooked-gear effort was conducted between late spring and early fall (May – October).

Samples of gonad tissue were collected from culled red grouper in years 2008-2013 and immediately fixed in 10% phosphate-buffered formalin. For histological analysis, ovarian tissue was fixed in 10% neutrally buffered formalin for 24 h, soaked in water for 1-2 h, and stored in 70% ethanol. Samples were embedded in glycol methacrylate, sectioned to 3–5-mm thickness, stained with periodic acid–Schiff's hematoxylin, and then counterstained with metanil yellow (Quintero-Hunter et al. 1991).

Gonadal analysis. Reproductive state, phase and histological indicators of red grouper were assigned following Lowerre-Barbieri et al. (2009) and Brown-Peterson et al. (2011) and criterion are outlined in Table 2. Histological indicators for female reproductive state are outlined in Table 3 and included: (1) oocyte developmental stages: primary growth (PG), cortical alveoli (CA), vitellogenic (Vtg1-3), and oocyte maturation (OM); (2) post ovulatory follicles (POFs); and (3) atresia. Secondary growth oocytes (SG) included CA, Vtg, and OM and fish with this level of development were considered mature (Lowerre-Barbieri et al., 2011b). To help distinguish immature from mature regenerating females, which both have only PG oocytes, the density of the PG population, thickness of ovarian wall and presence of muscle bundles extending from the ovarian wall into the ovarian lamellae were used as indicators (Lowerre-Barbieri et al., 2011b). Because oocyte maturation can take up to 16 h under the temperature regimes in which red grouper are spawning, the process of oocyte maturation was further broken down into: germinal vesicle migration (GVM), germinal vesicle breakdown (GVBD), yolk coalescence or clarification, and oocyte hydration (Jalabert 2005). Postovulatory follicles (POFs) were classified as either newly collapsed (recognizable by the size and appearance of the granulosa cells' nuclei) or 12 h or older based on POF size, organization, and elapsed time from peak spawning (Hunter & Macewicz 1985). Actively spawning females were considered to be those undergoing late OM, ovulation, or with fresh POFs (Tables 2 and 3). Only developing, spawning capable and regenerating reproductive phases were assigned to males, as active spawning can only be identified based on macroscopic analysis and there is no clear histological indicator to separate males late in the spawning season but still capable of spawning from those which are undergoing regression (Table 4).

Sex determination was based on histological analysis, as macroscopic assignments were inaccurate. Fish were considered male if only spermatogenic cells were present (i.e., no PG) or they had spermatozoa present (Trip et al., 2011). Similarly, sex was determined as female if there was nothing but female tissue or healthy SG oocytes were present. Parasitic nematodes were frequently observed in histological slides. Small cross sections of parasites looked similar to yolked oocytes undergoing atresia, with the exception of an external epithelial layer (Figure 2). Parasites occurred in both ovaries and testes and immature and mature females.

We defined fish undergoing sex change as transitional (no sex assigned) and broke this down into early and late transition. Early transition is defined as those fish with spermatagonia, spermatocytes, and some spermatids. Late transition includes proliferating amounts of male tissue with spermatids or later stages of spermatogenesis present (Table 5).

Data analysis. Our data set had 1153 fish with SL and only 679 fish with FL. The equation which resulted in the best FL estimate was:

Estimated FL=1.136556(SL)+15.903236 R^2=0.99

Because FL was the chosen length for the SEDAR, important reproductive parameters based on length are presented in SL and also converted to FL using the above equation.

Results / Discussion:

Sampling took place from 2008-2013 in the eastern Gulf of Mexico. The months sampled varied with year, with most fish collected during the months of March through November and one fish in December (Figure 3). A total of 1,180 fish were sampled for gonadal tissue. However, 22 of these samples were assessed as digestive tissue and removed from the data base. Of the fish with gonadal histology (n=1,157), there were; 917 females, 217 males and 23 fish undergoing transition from female to male.

Sex determination based on macroscopic analysis was not reliable, as of the 217 males all of them were categorized as females in the field. This is due to the sex change of the gonads, leaving testes with ovarian walls and often large numbers of primary growth oocytes (Figure 4). In addition, it was not possible to strip spawn males in the field to determine sex. Thus, there is a need to develop additional means of determining sex or to sex all fish using gonadal histology.

Male, female, and transitional sizes and ages showed a great deal of overlap (Figure 5). The average size of females was 398.4 mm SL (n=916) compared to 464.3 mm SL (n=215) for males and 415.0 mm SL (n=22) for transitionals (Fig. 5). Although these differences were significant (ANOVA, $F_{2,1054}$=33.08, $P < 0.0001$), individual variability and overlap in size was high, with females ranging in size from 130 to 677 mm SL and males from 179 to 709 mm SL. On average, males were older (mean=7.1, SD=2.6) than females (mean= 5.7, SD=2.1) However, as with size, there was a great deal of individual overlap with females ranging from 1 to 14 years and males from 1 to 16 years. The mode in male ages was age 6 (Figure 6), the same as the female mode and a year younger than the age at which males first became prevalent in the 1960's (Moe, 1969).

Although our original sampling focused on females, 19% of those collected based on a field assessment of female were in fact male. This proportion of males is fairly similar to sex ratios reported in other studies (Moe, 1969, Brulé et al., 1999), but it cannot be ruled out that this is an underestimate. A higher proportion of males (28%) was reported in the last SEDAR (Fitzhugh et al., 2006).

Spawning capable females were collected from March through July, with the majority occurring in March through June (Fig. 3). This is similar to previous reports of spawning seasonality in this species (Moe, 1969, Burgos et al., 2007). In comparison at least some spawning capable males occurred in all of the months sampled (March through November). However, regenerating males occurred from July through October, also suggesting most spawning occurred in March through June. Individual spawning periods appear to be quite asynchronous, given that some regressing or regenerating females occurred in each month sampled. Within the spawning season (March-June), the size range of females with spawning indicators or secondary growth oocytes (used as an indicator of maturity) were similar, although there were more spawning

females in larger size classes (Figure 6). Regressing females (atretic) were more prevalent in smaller size classes (mean SL=402 mm SL). However, in mature females the mean size of those with no secondary growth oocytes (mean SL=413 mm), was similar to those with secondary growth oocytes (mean SL= 437 mm), and those with spawning indicators (mean SL=433 mm; Figure 7).

Most transitioning occurs at the end of spawning season or later. We found a total of 23 fish undergoing transition from female to male (age range 2-7) collected in the months of June through November. The highest numbers of transitionals occurred in July and August, but with a second group occurring in October. A fish considered in early transition was collected in June. This fish had small pockets of proliferating male tissue throughout the lamellae, while it still retained yolked oocytes and presumably was capable of spawning as a female.

Immature females ranged in size from 130 to 416 mm SL, with a mean of 269 mm SL and overlapped with the size range of mature females (with secondary growth oocytes: cortical alveoli or more developed), which had a range of 164 to 677 mm SL and a mean of 411 mm SL (Figure 8). For data from the spawning season (March through June), estimated size at 50% mature was 258 mm SL (SE=5.43) and 332 FL (SE=6.36). The estimated age at 50% mature was 2.96 (SE=0.25). The size at 50% males was 609.3 mm SL (SE=22.3) and 742.6 FL (SE=26.1). Age at 50% male was 11.5 years. These estimates of maturity and 50% male are similar to those previously used. We found no spatial trend in small males with sampling location (Figure 9). Spawning capable and spawning females were more prevalent from Tampa Bay north. However, they were also more prevalent in hook and line sampling which was conducted primarily in this area (Fig. 10).

Table 1. Summary of reproductive samples collected by general gear type in the eastern Gulf of Mexico. See methods section in the text for a description of gear types and surveys comprising each category listed.

Year	Trawls	Traps	Hooked Gear	Total
2008	0	24	4	28
2009	12	15	39	66
2010	0	8	60	68
2011	21	59	0	80
2012	0	83	155	238
2013	62	292	323	677
Total	95	481	581	1157

Table 2. Ovarian classification and terms based on histological analysis.

Reproductive state		Phase	Histological indicators	Signficance
Immature	Nonspawn-ing	Immature	Only oogonia and primary growth oocytes, including chromatin nucleolar and perinucleolar oocytes. Usually no atresia.	Virgin that has not yet recruited to the spawning population.
Mature	Nonspawn-ing	Developing	Cortical alveolar and sometimes early yolked oocytes. No evidence of POFs. Some atresia may be present.	Environmental signals have triggered development, but fish are not yet developed enough to spawn.
Mature Spawning population	Spawning-capable	Spawning-capable	Yolked oocytes. May be some atresia.	Fish developed enough to spawn.
	Spawning	Spawning	Oocyte maturation, hydration or POFs.	Fish with indicators of spawning activity.
		Subphases:		
		Imminent	Early OM (GVM with little yolk coalescence)	Will spawn in 14 h.
		Active	1. Late OM (completed GVM or GVBD with yolk coalescence and partial to full hydration), 2. Ovulation 3. Newly-collapsed POFs	Spawning +/- 2 h.
		Recent	POFs (12-36 h old)	Spawned within 2 d.
Mature	Nonspawn-ing	Regressing	A high percentage of yolked oocytes undergoing atresia (alpha and beta).	Cessation of spawning.
	Nonspawn-ing	Regenerating	Only primary growth oocytes present, including chromatin nucleolar and perinucleolar. Muscle bundles, enlarged blood vessels, thick and/ or convoluted ovarian wall, and gamma or delta atresia may be present.	Sexually mature, reproductively inactive. Most common outside of the spawning season.

Table 3. Histological basis for reproductive phases in female red grouper, *Epinephalus morio.*

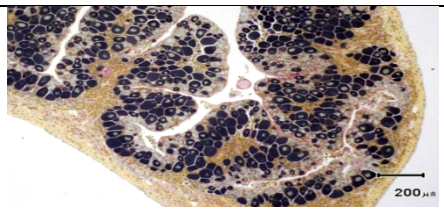
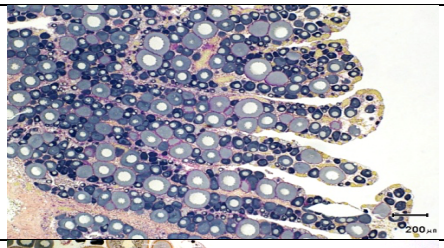
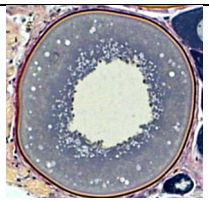
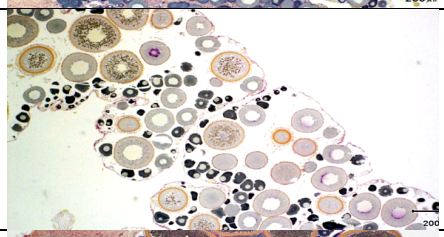
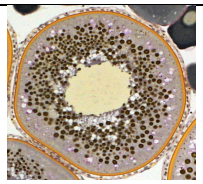
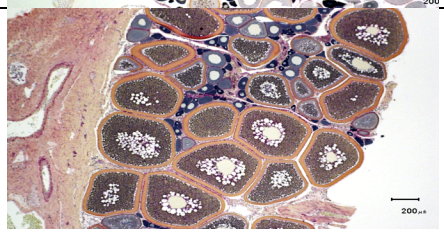
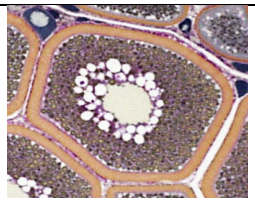
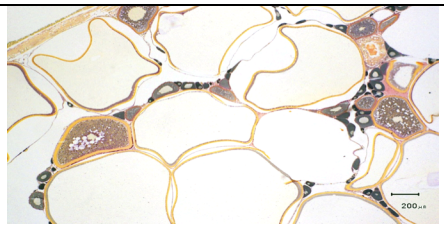
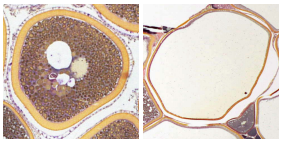
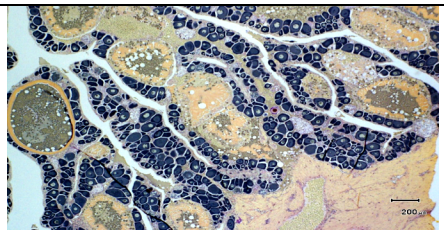
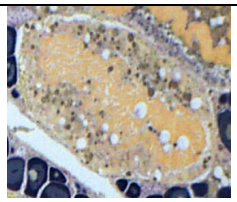
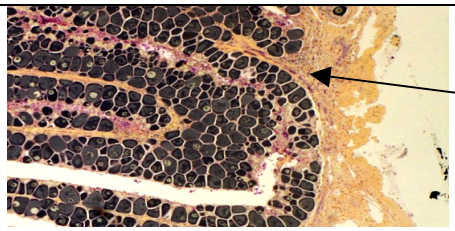
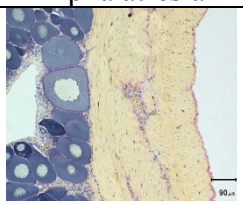
Ovarian Cross Section	Phase Characteristics	Most advanced oocyte or key histological indicator
	**Immature Phase** • Only oogonia & PG • No muscle bundles. • Thin ovarian wall. • Well-organized lamellae.	 Perinucleolar
	***Early Developing – Sub phase*** • PG and CA(cortical alveoli) oocytes only • Can be some atresia	 Cortical alveolar
	**Developing** • Primary growth, cortical • alveolar, vitellogenic • oocytes stages 1 & 2 • Atresia can be present	 Vitellogenic 2
	**Spawning Capable** • Completed vitellogenesis (Vtg3) • Can have post ovulatory follicles present	 Vitellogenic 3
	**Actively Spawning subphase** • Late oocyte maturation (GVM, GVBD, & hydration) • Or ovulation • Fresh POFs	 GVM Hydration
	**Regressing** • Most vtg oocytes atretic; 1st indicator alpha atresia discontinuous zona pellucida • PG and CA oocytes present	 Alpha atresia
	**Regenerating** • Oogonia and PG oocytes • Muscle bundles often • Thick ovarian wall • May have late stage atresia	 Thick ovarian wall

Table 4. Histological basis for reproductive phases in male red grouper, *Epinephalus morio.*

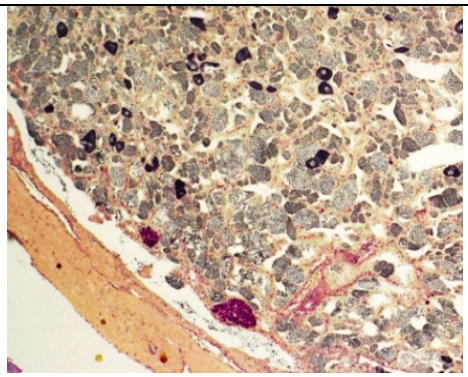
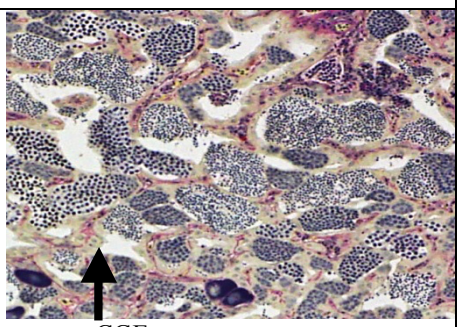
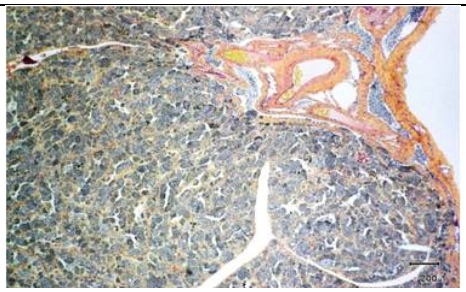
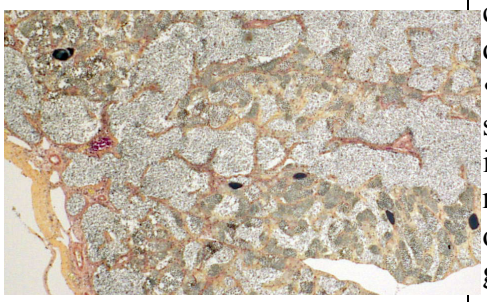
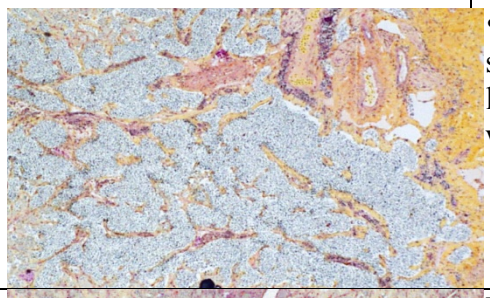
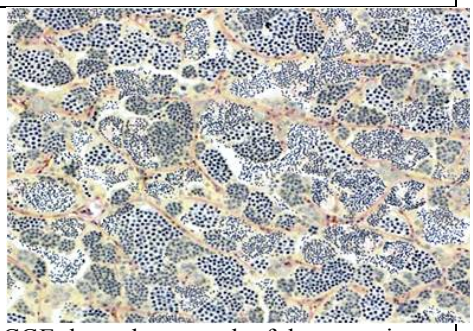
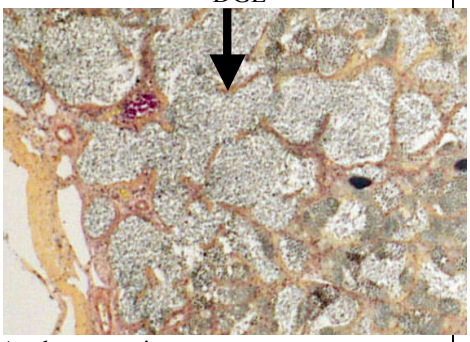
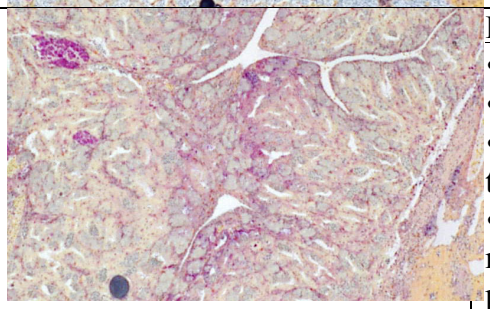
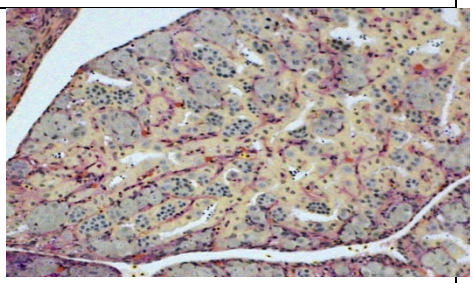
Teste Cross Section	Phase Characteristics	Key histological indicator
	**<u>Developing</u>** • Spermatocysts evident along lobules • Spermatogonia(Sg), spermatocytes(Sc), spermatids(St) and • Sz cannot be present in lumen of lobules or ducts, can occur within spermatocysts	 CGE Continuous germinal epithelium (CGE)
  	**<u>Spawning Capable</u>** • Sz in lumen of lobules and/or sperm ducts. • All stages of spermatogenesis can be present. • Germinal epithelium (GE) can be continuous(CGE) or discontinuous(DGE) • Actively spawning subphase can be identified only macroscopically based on if milt released with gentle pressure on abdomen • In hermaphroditic species, sperm ducts are located in the ovarian wall.	 CGE throughout much of the testes in the early part of the spawning season. DGE  As the spawning season progresses, DGE increases and Sg are less common. Sz fill the lumen of lobules and sperm ducts.
	**<u>Regenerating</u>** • No spermatocysts • Often no lobule lumen • Proliferation of Sg throughout testes • Small amount of residual Sz can be present	 Continuous GE throughout

Table 5. Histological indicators of fish undergoing transition in red grouper, *Epinephalus morio.*

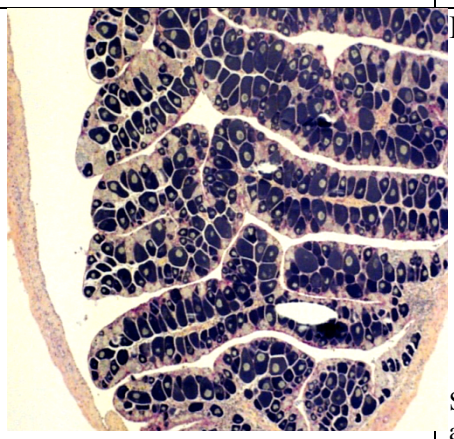
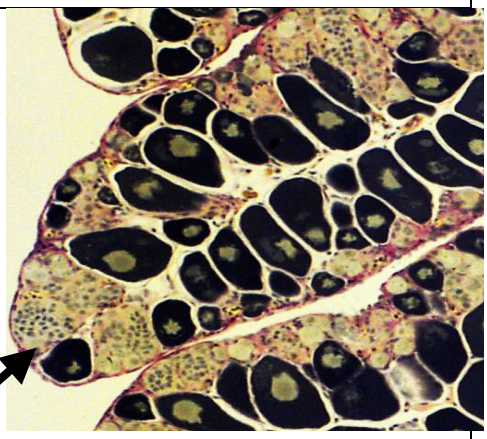
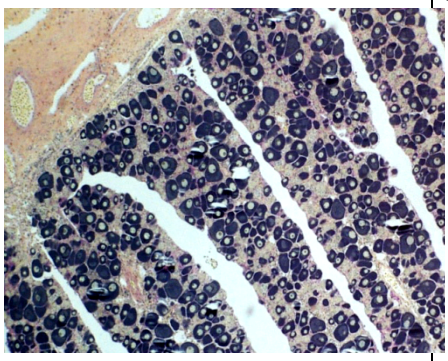
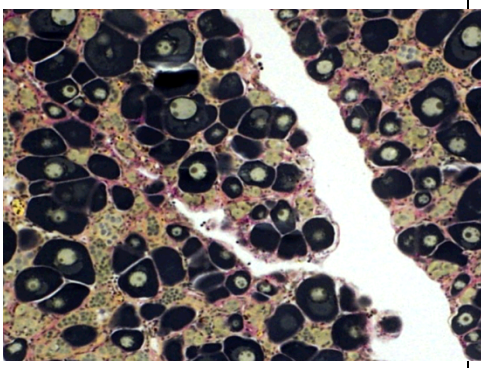
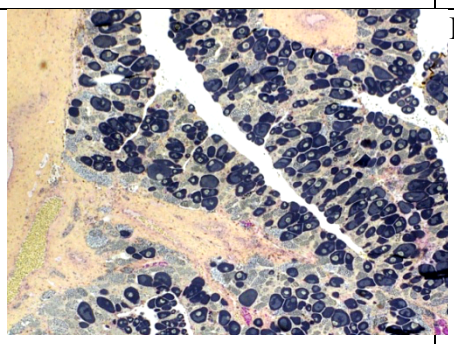
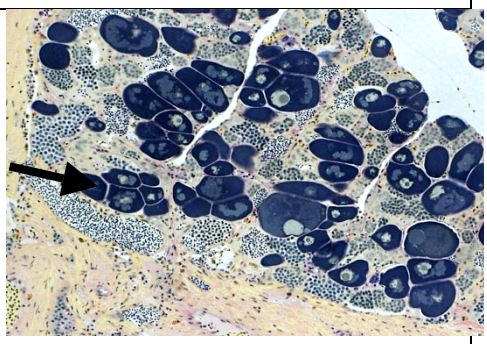
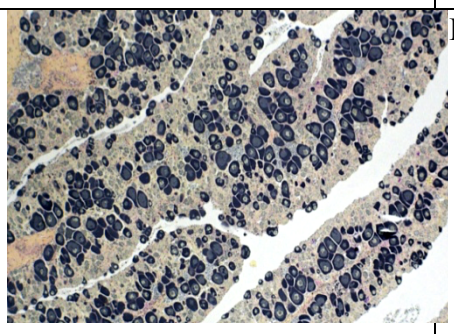
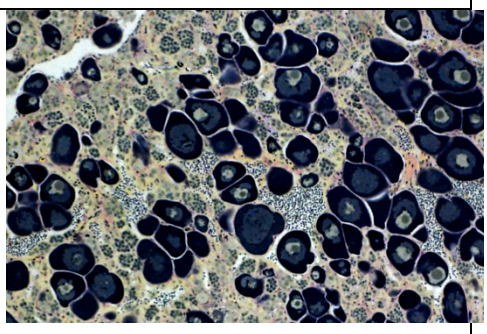
Cross Section	Transition description	Key histological indicator
	Early transition • Spermatagonia (Sg) present • Spermatocytes (Sc) most developed prevalent male tissue • Primary growth (PG) oocytes • Some spermatids (St) may be present Sg and Sc are developing all along periphery of lamellae.	
	Early transition • Sg and Sc are prolific and have developed throughout the lamellae. Numbers of PGs are decreasing.	
	Late transition • St are the most developed tissue	
	Late transition • St throughout lamellae • If Sz present, male • PG may still be abundant in a spawning capable male	

Figure 1. Locations where red grouper histology samples were collected in association with various surveys (from left to right: trawling, trapping, hook and line) conducted by FWC from 2008 – 2013.

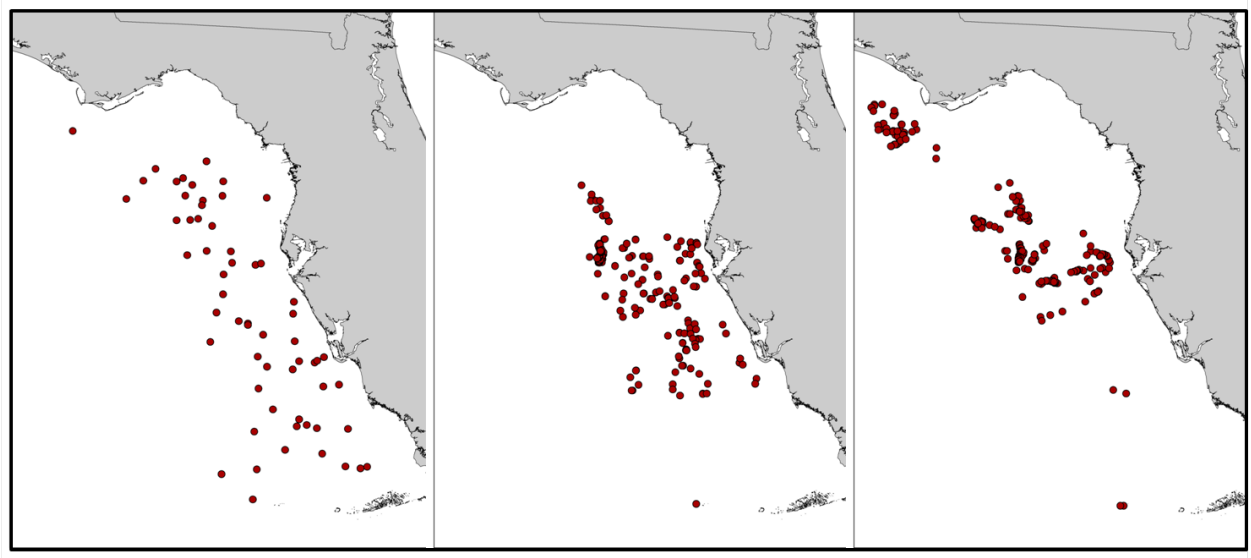


Figure 2. Parasites within the ovarian luman of an immature red grouper (top) and within the ovarian lamellae of a regenerating red grouper (bottom).

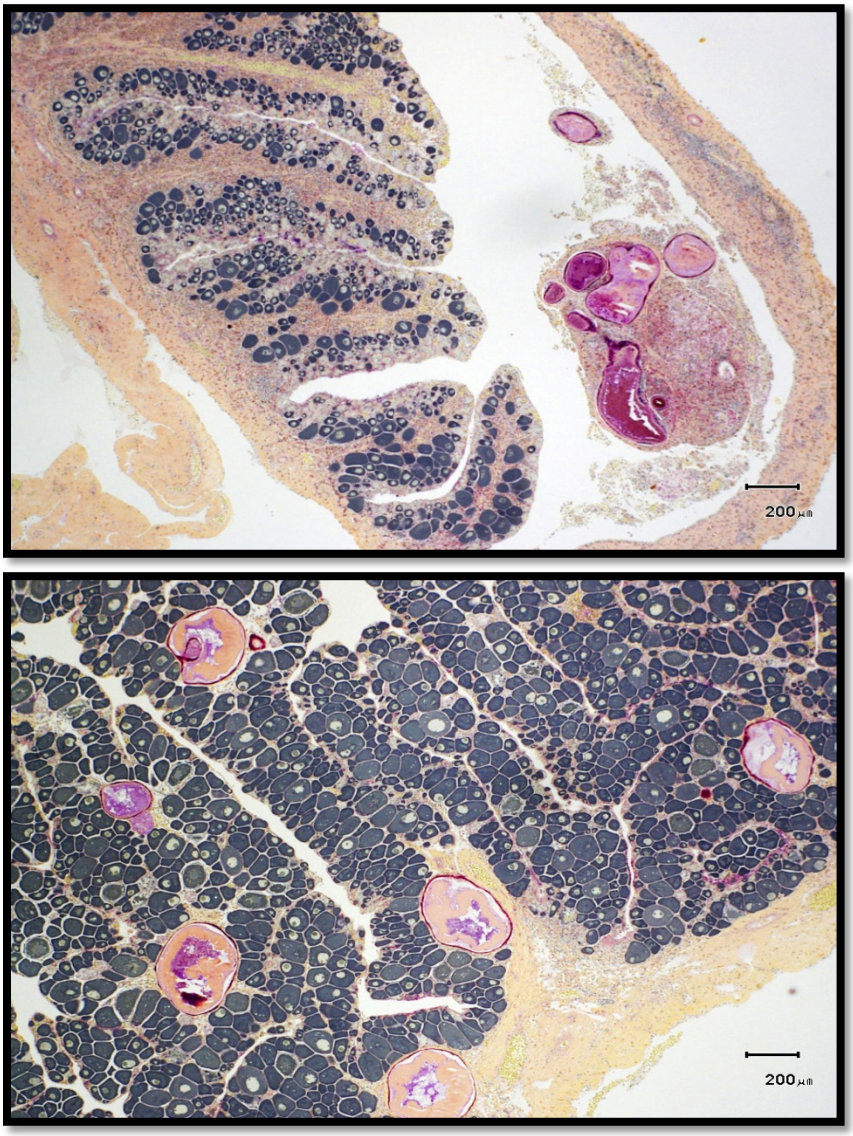


Figure 3. The temporal distribution of reproductive phases. Spawning capable females occurred primarily in the months of March through May.

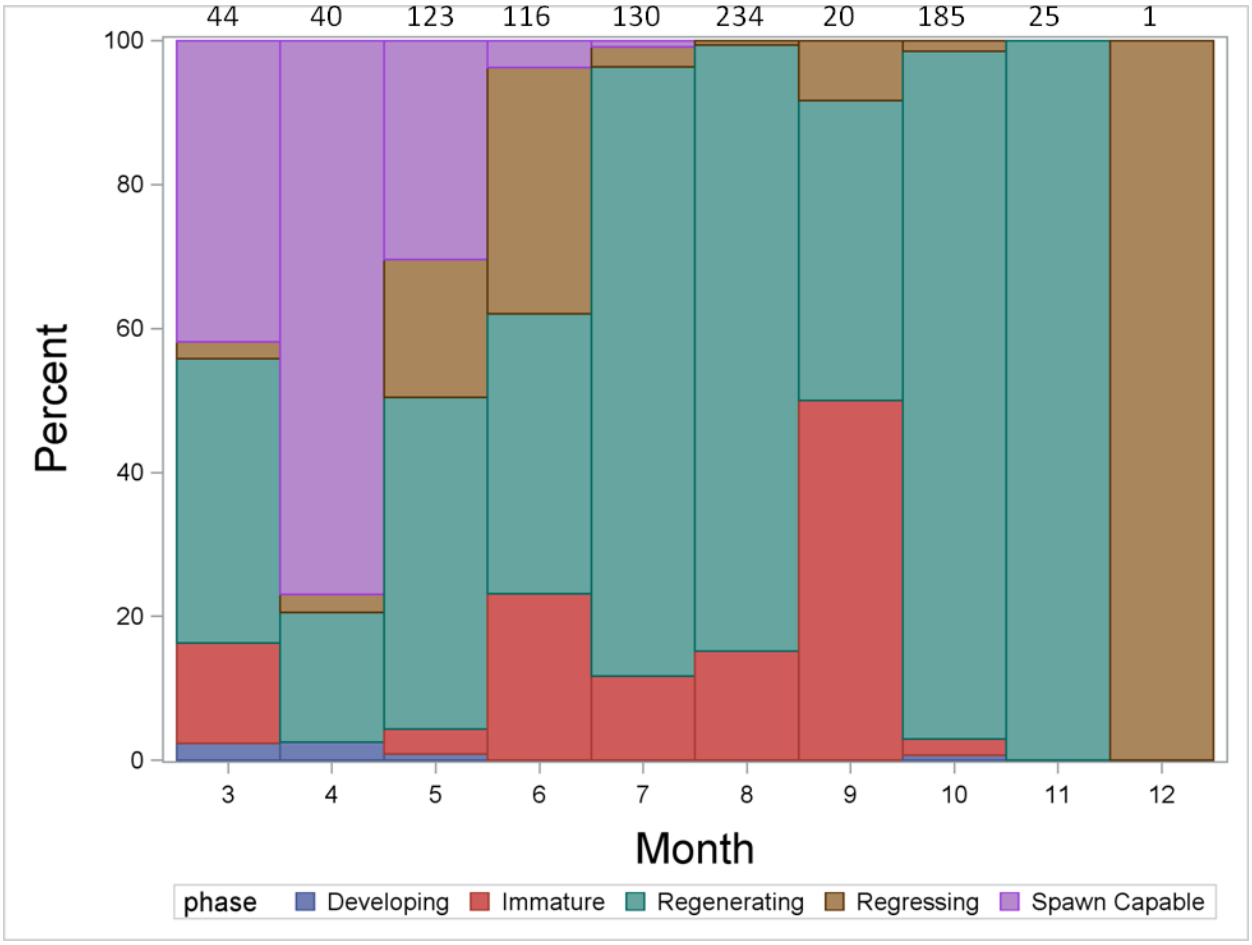


Figure 4. Macrosopic and histological appearance of red grouper (A) female regenerating and (B) male, recently transitioned.

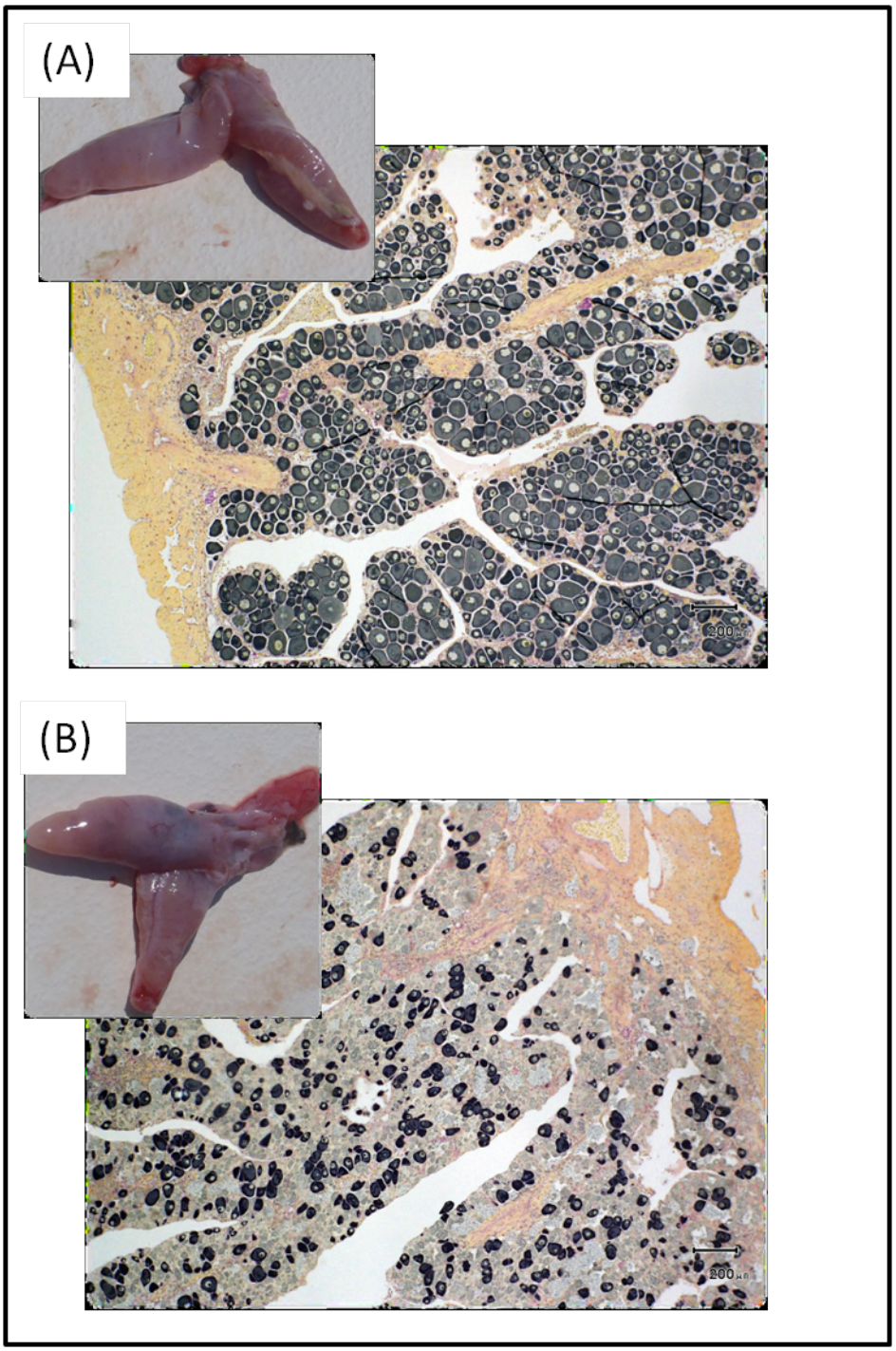


Figure 5. Age distribution of red grouper by sex (top) and by size (bottom).

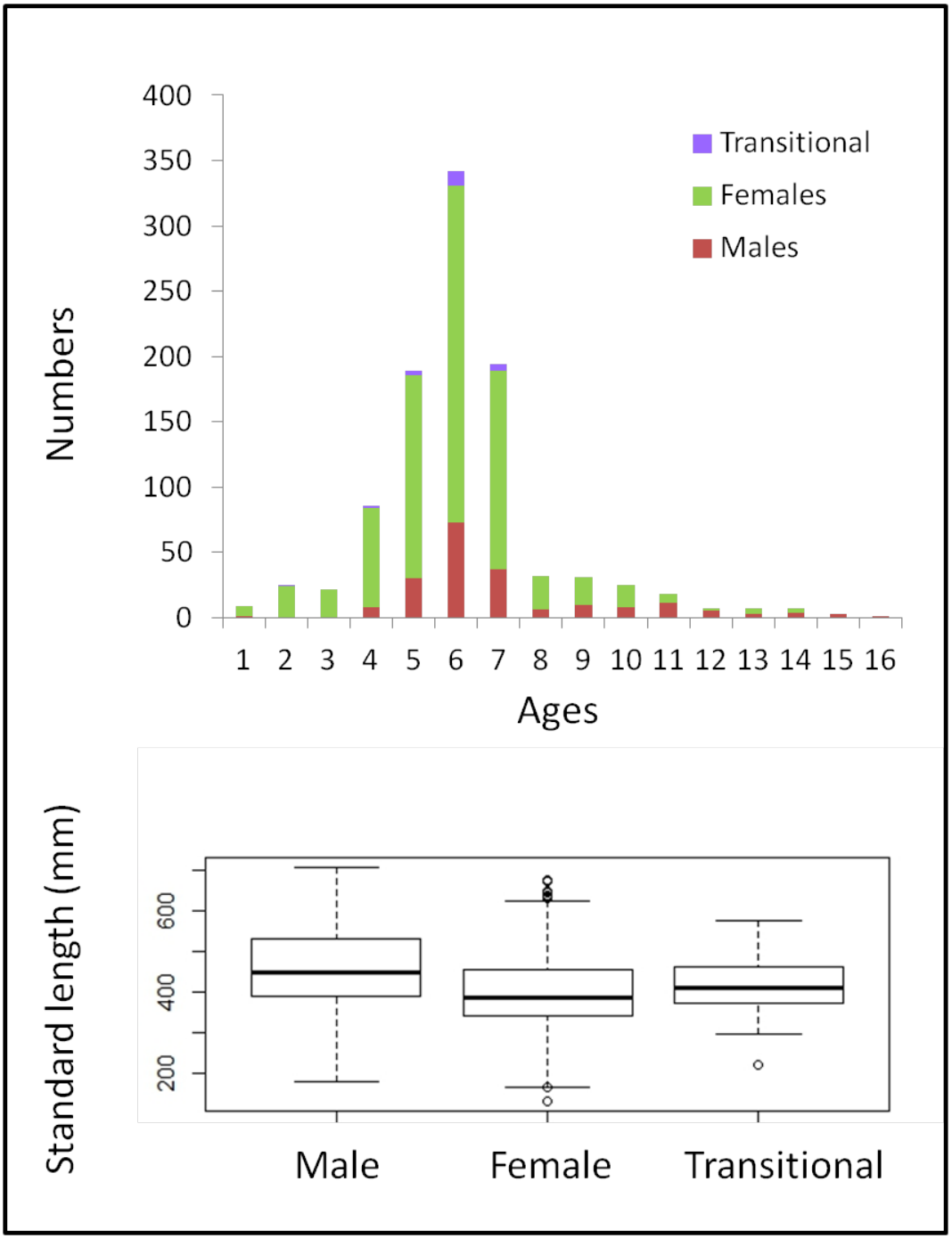


Figure 6. The size distribution (standard length) of females from within the spawning season plotted by histological indicator: PG/immature (no signs of prior development), PG/mature (indicators of previous development), secondary growth oocytes (CA and Vtg, indicators of maturity), atretic, indicating the majority of secondary growth oocytes were being resorbed (i.e., cessation of spawning), and fish with spawning indicators.

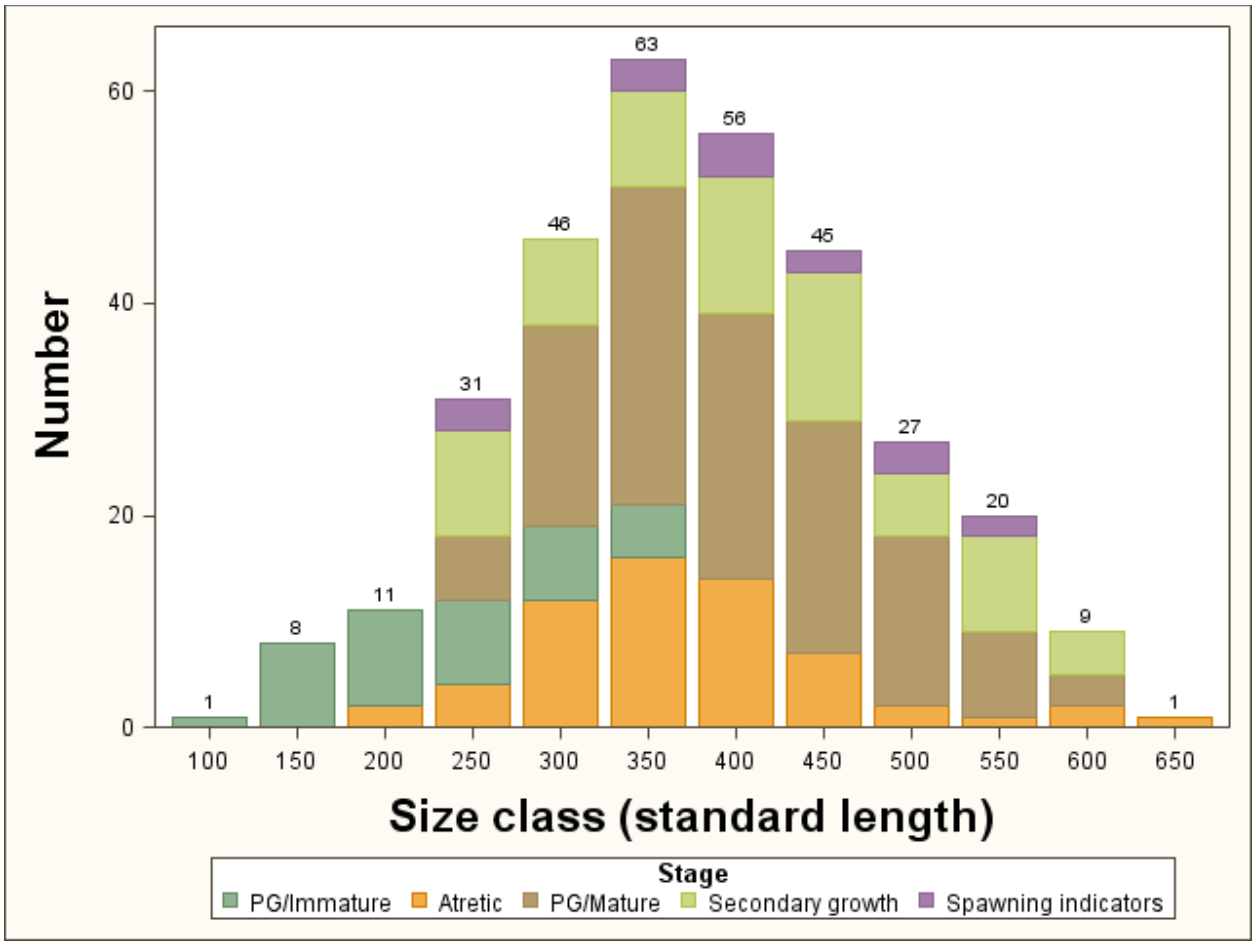


Figure 7. The mean, 25%/75% quantiles, and range of female (standard length) by histological indicator within the spawning season. PG/immature (no signs of prior development), PG/mature (indicators of previous development), secondary growth oocytes (CA and Vtg, indicators of maturity), atretic, indicating the majority of secondary growth oocytes were being resorbed (i.e., cessation of spawning), and fish with spawning indicators.

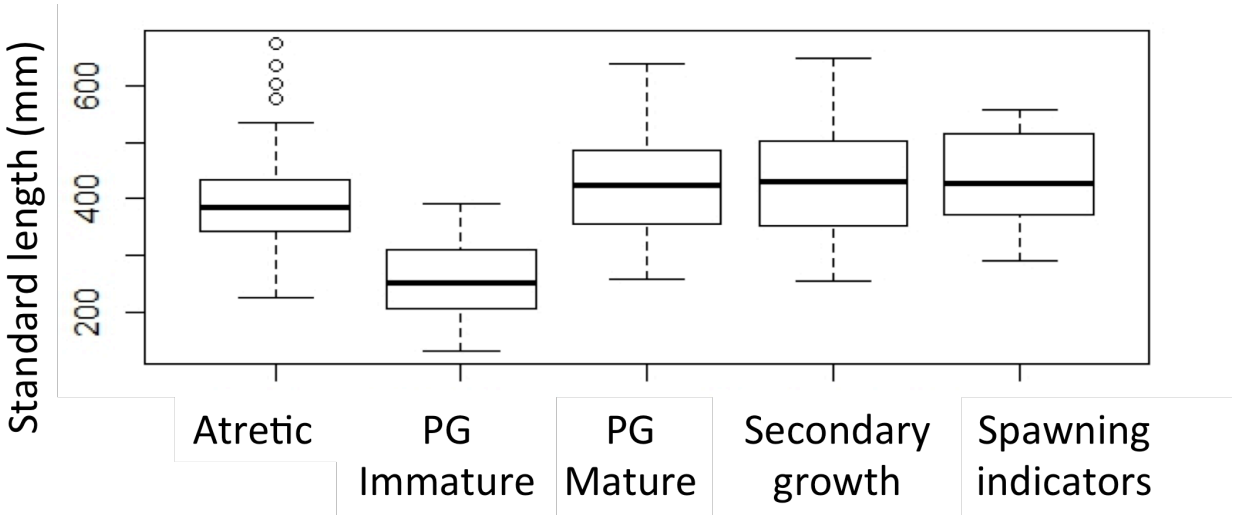


Figure 8. The size distribution of immature and mature females and the sample size (n=914).

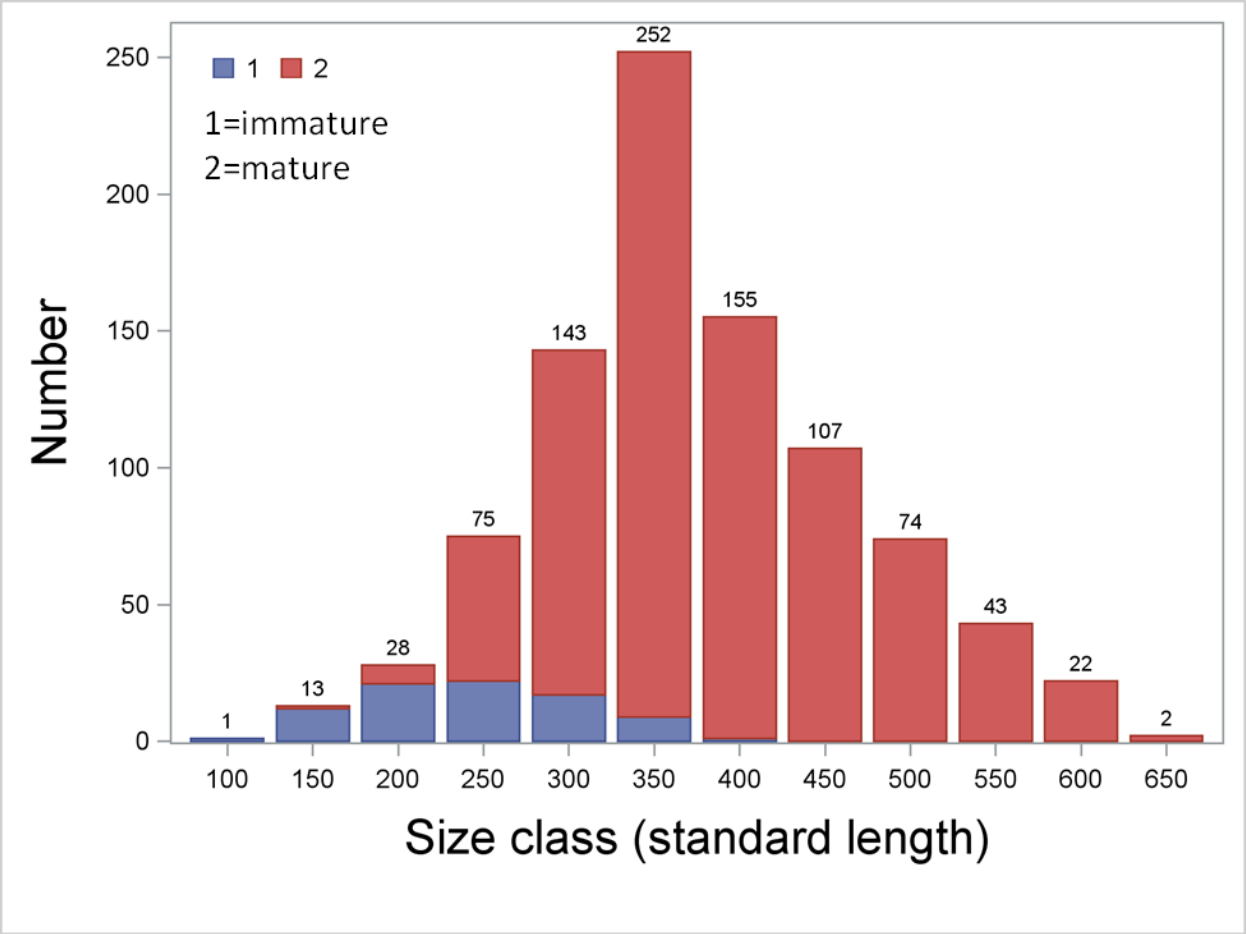


Figure 9. Distribution of male collection site by size class: large = > 500 mm SL and smaller < 500 mm SL.

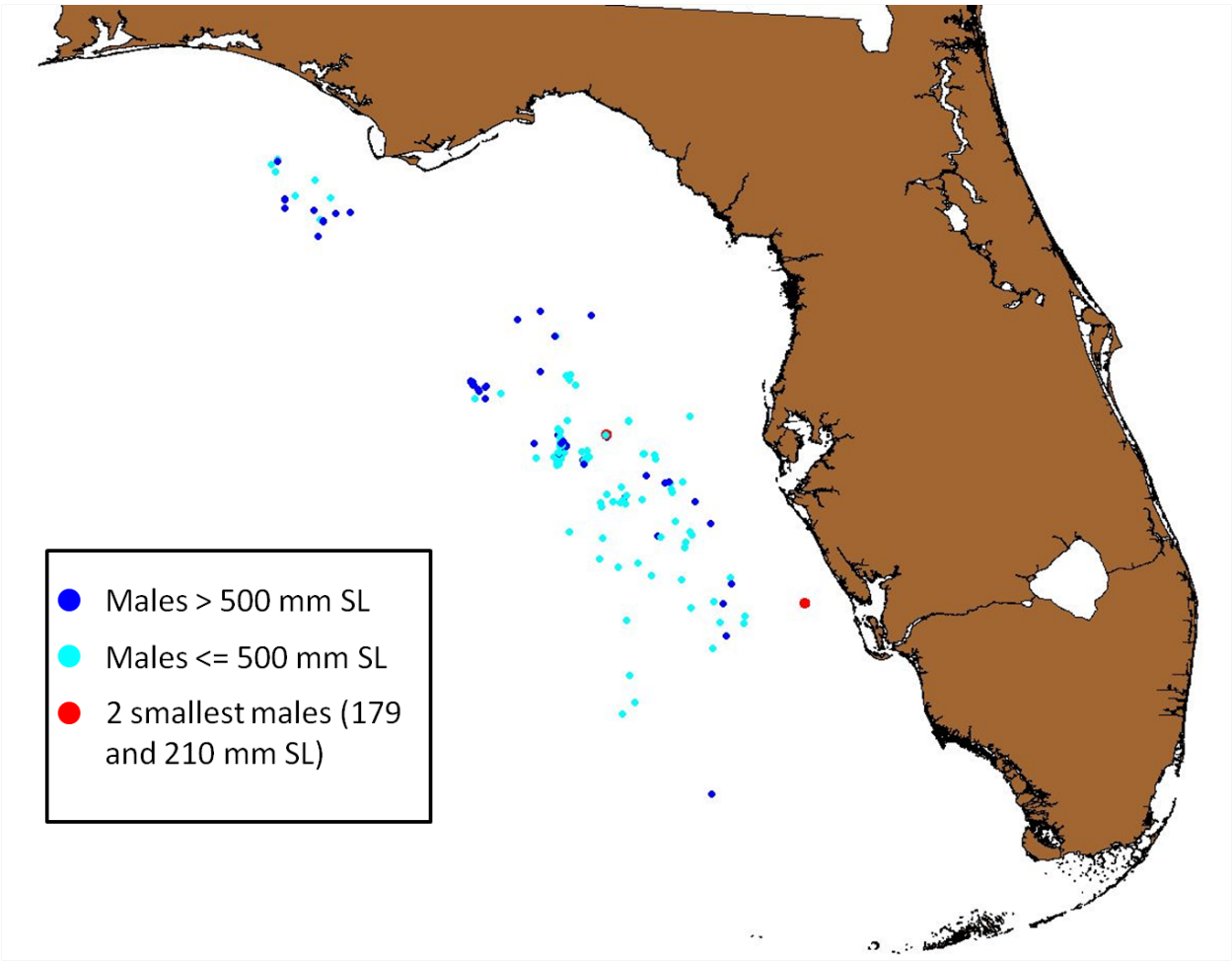
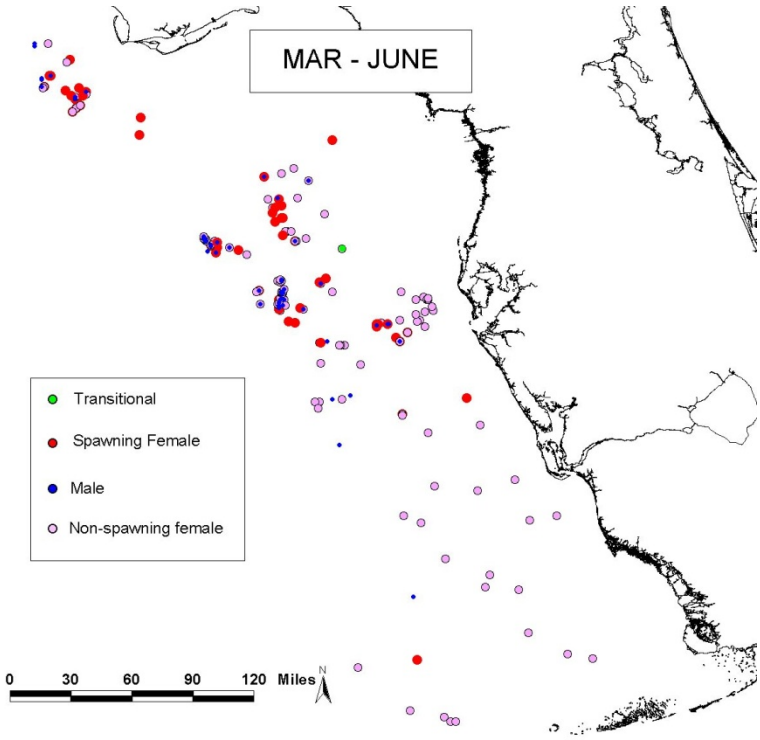


Figure 10. The spatial distribution during the spawning season (March through June) of males, fish undergoing sex change (transitionals), spawning capable females (spawning or fully yolked), and non-spawning females (developing (n=3) or regressing or regenerating).



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