Summer 8-2016

Growth and Reproduction of Southern Flounder (*Paralichthys lethostigma*) in the North-Central Gulf of Mexico

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GROWTH AND REPRODUCTION OF SOUTHERN FLOUNDER (*PARALICHTHYS LETHOSTIGMA*) IN THE NORTH-CENTRAL GULF OF MEXICO

by

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A Thesis
Submitted to the Graduate School
and the Department of Coastal Sciences
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

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August 2016

Published by the Graduate School

ABSTRACT

GROWTH AND REPRODUCTION OF SOUTHERN FLOUNDER (*PARALICHTHYS LETHOSTIGMA*) IN THE NORTH-CENTRAL GULF OF MEXICO

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Southern Flounder *Paralichthys lethostigma* is the most commonly harvested flatfish in the north-central Gulf of Mexico (GOM) and supports a major inshore recreational fishery, yet knowledge of the species' life history is greatly limited. The objective of this research was to describe the growth and reproduction of Southern Flounder in the Mississippi stock. Fish were collected during September 2014 to March 2016 using primarily recreational fishing techniques. Otoliths (n = 313) were sectioned to estimate age, and multiple length-at-age models were fit to total length (TL, mm) and age estimate (y) data. Gonadal tissue samples (n = 221) were preserved for histological analysis and used to classify reproductive phases. Length-at-age model fit was evaluated using Akaike information criteria, revealing that the three-parameter von Bertalanffy growth function best described the female-specific data (L_∞ = 509 mm, k = 0.70 y^{-1}, t_0 = -0.46 y). By fitting a logistic model to binomial maturity data, the mean length-at-50% maturity was estimated as 303 mm TL and mean age-at-50% maturity was estimated as one year for females. Histological indicators and gonadosomatic index (GSI) data were used to estimate that the spawning season lasts from November to January, and to classify Southern Flounder as batch spawners. These results will inform future stock assessments and management decisions for the GOM Flounder fishery.

ACKNOWLEDGMENTS

Funding for this thesis research was provided by the Mississippi Tidelands Trust Fund Program, administered by the Department of Marine Resources, and awarded to S. D. Clardy and R. T. Leaf. I am thankful for the financial support to attend professional meetings from The University of Southern Mississippi American Fisheries Society student subunit and the Mississippi Chapter of the American Fisheries Society. I would like to thank my advisor, Robert T. Leaf, and committee members, Nancy J. Brown-Peterson and Mark S. Peterson, for their immense contribution to the development of my thesis. The University of Southern Mississippi Marine Education Center and the Center for Fisheries Research and Development staff provided constant support with field sampling and community outreach for the project. Specifically, I thank Samuel D. Clardy for coordinating sampling trips and Gary Gray for his technical expertise in otolith-based aging methods. This research would not have been possible without the many individuals who assisted me in collecting fish for the project, including charter boat captains and countless recreational anglers. Thanks to the Project INSPIRE student interns for volunteering their time to assist with laboratory sample processing. Finally, thank you to my labmates, Dave, Cody, Grant, and Taylor, for your source of knowledge and humor.

DEDICATION

To my support system in family and friends, a heartfelt thank you for your unconditional encouragement throughout my graduate career. Thank you to my parents Doris and David, and Austin, for supporting me in moving to the Mississippi coast. Your long-distance love and support means more than I can express. Thanks to my fellow graduate students at the Gulf Coast Research Lab for making me laugh and keeping me motivated. In particular, Page and Nour, thank you for giving me a home. I will always be grateful to the Ocean Springs community for making my time here memorable.

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CHAPTER I – BACKGROUND

Southern Flounder *Paralichthys lethostigma* is the most commonly harvested flatfish species that occurs in the north-central Gulf of Mexico (GOM) (Hensley and Ahlstrom 1984). Southern Flounder are distributed as far north as Albermarle Sound, North Carolina on the U.S. Atlantic coast and throughout the GOM to northern Mexico, and exhibit a geographic break around the southernmost Florida peninsula (Hensley and Ahlstrom 1984, Enge and Mulholland 1985, Reagan and Wingo 1985). However, the Atlantic and GOM populations are separated geographically around the southernmost Florida peninsula. There is evidence for genetic distinction between the Atlantic and GOM Southern Flounder populations, and some small-scale genetic differences have been reported within the GOM (Blandon et al. 2001, Anderson and Karel 2012).

Southern Flounder are a euryhaline, estuarine-dependent species that exhibits seasonal migration patterns (Deubler 1960, Etzold and Christmas 1979). Southern Flounder migrate to offshore waters in winter months and spawn pelagic eggs that undergo hydration, a process that makes the eggs buoyant for effective transport to nursery habitats (Benson 1982). Larvae undergo metamorphosis and settlement in lower salinity estuarine waters during late winter and spring months, where feeding and growth occurs (Stokes 1977, Shepard 1986, Ditty et al. 1988). Southern Flounder also inhabit freshwater environments, which is supported by otolith microchemistry analyses in the Mobile-Tensaw River Delta of Alabama and in Texas coastal waters (Lowe et al. 2011, Farmer et al. 2013, Nims and Walther 2014). Residency patterns of Southern Flounder have been studied in the Atlantic using a tagging approach, and results indicated limited movement during winter estuarine residency with extensive movement of larger

individuals during spawning migrations to the southeastern U.S. continental shelf (Craig et al. 2015). However, little is known about the spawning habitats of Southern Flounder in the GOM.

The Southern Flounder stock is a valuable marine resource in the GOM supporting both a recreational and commercial fishery. Although Southern Flounder and Gulf Flounder *Paralichthys albigutta* are managed as a single stock, Southern Flounder is the more abundant of the two species harvested from Alabama to Texas in the GOM (Adkins et al. 1998). Flounder species are primarily harvested recreationally using hook-and-line fishing or gigging (GSMFC 2015) with the Gulf-wide recreational harvest averaging over 400,000 kg per year for the past decade (NOAA National Marine Fisheries Service, 2015). However, long-term declines in population size have been observed in Texas between 1975 and 2008 (Froeschke et al. 2011). Despite the economic value of this species and evidence for overfishing, life-history information for Southern Flounder in the north-central GOM is limited. An understanding of life history improves the ability to manage a population sustainably (Adams 1980, Winemiller and Rose 1992). Further research on the life history of Southern Flounder is therefore beneficial for informing management of the stock.

The objective of this research is to describe the growth (Chapter II) and reproduction (Chapter III) of Southern Flounder in the north-central GOM followed by a synthesis of management considerations (Chapter IV). A sampling effort was conducted using multiple gear types to collect fish between September 2014 to March 2016. Size measurements and otoliths were collected from each fish to estimate female-specific length-at-age parameters. Reproductive tissue samples were processed using histological

techniques to estimate age- and length-at-maturity and to describe spawning seasonality for female Southern Flounder. Finally, the results of this study were compared to results reported in previous studies of Southern Flounder growth and reproduction. The knowledge gained from this research will improve the understanding of Southern Flounder life history and the ability to manage the north-central GOM stock.

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CHAPTER II – AGE AND GROWTH DYNAMICS OF SOUTHERN FLOUNDER

Introduction

Southern Flounder *Paralichthys lethostigma* supports a major inshore recreational fishery in Mississippi with mean annual harvests over 90,000 kg for the past decade (NOAA National Marine Fisheries Service, 2016). Despite the economic value of the Southern Flounder fishery, age and growth information for Southern Flounder in the north-central Gulf of Mexico (GOM) is limited. Growth is a fundamental life-history characteristic that influences population dynamics, and therefore, an understanding of growth is necessary for fisheries management (Adams 1980, Denney et al. 2002). The Southern Flounder fishery is independently managed by state agencies, and currently there is a 12-inch (305 mm) minimum size limit enacted in Mississippi (GSMFC 2015). Characteristics of Southern Flounder age and growth have not been described in Mississippi waters. Thus, there is a need for understanding the local population dynamics of Southern Flounder.

Previous life-history studies have described the growth of Southern Flounder in the Atlantic (Wenner et al. 1990, Fitzhugh et al. 1996) as well as the Gulf coast of Texas (Stunz et al. 2000, Glass et al. 2008), Louisiana (Fischer and Thompson 2004), and Florida (Nall 1979, Frick 1988). Based on past research, Southern Flounder exhibit relatively fast growth and has a maximum reported age of ten years (Nall 1979). The longest mean theoretical length (L_∞) for Southern Flounder was estimated as 1461 mm standard length in Florida (Nall 1979). However, this estimate is far greater than the longest observed length from any location in the GOM. Southern Flounder also exhibit sexually dimorphic growth with faster growth rates, older maximum ages, and greater

maximum lengths reported for females (Wenner et al. 1990, Stunz et al. 2000, Fischer and Thompson 2004). Consequently, females make up the primary harvest of the fishery (Takade-Heumacher and Batsavage 2009). Female-specific estimates of L_∞ for Southern Flounder reported in Texas (Stunz et al. 2000) and Louisiana (Fischer and Thompson 2004) are based on validated age estimates and range from 483 to 556 mm total length.

Growth parameter estimates for Southern Flounder are spatially variable within the GOM, although there are no apparent patterns in spatial variability. Midway et al. (2015) used a hierarchical Bayesian model fitting process to show that growth parameter estimates are variable within GOM states, and hypothesized that differences are due to spatially-distinct environmental conditions. Although there is no evidence for independent stocks within the GOM, small-scale genetic structuring has been reported (Blandon et al. 2001). However, because Southern Flounder is a fast-growing, estuarine-dependent species, growth may be more influenced by environmental variability than genetic differences (Midway et al. 2015). The results reported by Midway et al. (2015) indicate that local estimates of growth parameters should be used to assess the stock of interest and inform state-level management. The length-at-age relationship is necessary to inform age-structured stock assessment models (Quinn and Deriso 1999), yet current estimates of length-at-age parameters are lacking for Southern Flounder in the north-central GOM.

The Southern Flounder length-at-age relationship has been described previously using the von Bertalanffy growth function (Nall 1979, Frick 1988, Wenner et al. 1990, Stunz et al. 2000, Fischer and Thompson 2004). However, this model is not always the most appropriate model for describing ontogenetic growth of a given species (Cailliet et

al. 2006). The approach of fitting multiple models and comparing candidate models can help reduce model selection uncertainty and improve parameter estimates (Burnham and Anderson 2002, Katsanevakis 2006). Multi-model approaches for describing the length-at-age relationship are widespread in the marine fisheries literature (Mercier et al. 2011, Harry et al. 2013, Higgins et al. 2015, Dippold et al. 2016), but the approach has not been widely used for describing Southern Flounder growth (Fischer and Thompson 2004, Midway et al. 2015).

The overall goal of this research is to describe the age and growth of Southern Flounder in the north-central GOM. Specifically, the following objectives were developed: 1) to validate the formation of annuli in Southern Flounder otoliths and evaluate factors that influence otolith growth; 2) to describe the female-specific length-at-age and weight-at-length relationships for Southern Flounder; 3) to compare growth parameter estimates to published growth parameter estimates for Southern Flounder; and 4) to evaluate seasonal changes in relative condition of Southern Flounder. Accomplishing these objectives will allow for a better understanding of Southern Flounder life history and enable better management of the north-central GOM stock.

Methods

Southern Flounder were collected and processed from September 2014 to February 2016 in the north-central GOM, primarily within Mississippi waters (Figure 1). The most common sampling methods employed were gigging and hook-and-line fishing, but fish were also collected using trawls, gill nets, bow fishing, crab traps, seines, and cast nets. Additional fish were obtained from local recreational fishing tournaments and

from fishery-independent research surveys. Fish were immediately placed on ice following collection and processed in the laboratory.

Each fish was measured for total length (TL, mm), standard length (SL, mm) and wet body weight (g), and its otoliths removed for aging. The paired sagittal otoliths were removed from each fish by exposing the brain cavity with a transverse cut. Otoliths were rinsed to remove membranous tissue and stored in a labeled envelope. The left sagittal otolith was processed for age estimation from a subsample of fish (n = 367) selected to represent all 50 mm TL size bins collected in this study. The otolith was embedded in a mold with Epoxicure resin and allowed to harden for a minimum of 24 hours. Once the resin hardened, the resin block was marked to target the otolith core and several sections were cut at a thickness of about 0.4 mm with a Buehler diamond blade saw. Otolith sections were then polished to increase the visibility of annuli and mounted on slides with Crystalbond and Flo-Texx mounting mediums. To evaluate a sufficient sample size of otoliths processed for age estimation, I used a resampling procedure where the two-parameter von Bertalanffy growth function (VBGF) was fit to the age estimate and TL data with each iteration increasing the sample size by n = 1. The coefficient of variation was estimated for both parameters with increasing sample size.

Age estimates were determined using otolith annuli counts and the frequency of annuli deposition was validated using marginal increment analysis (MIA). Annuli were counted from images taken at 2× to 5× magnification under transmitted light with a Stemi 2000-C microscope. Two independent readers reported an age estimate by counting fully-formed annuli, and the age estimate was excluded from analysis if an agreement was not reached between readers. The percent agreement (PA) was calculated to compare

assignments of age estimates, and the standard deviation (SD) and coefficient of variation (CV) were calculated. The otolith radius (µm), annuli width (µm), and translucent area formed on the outer edge margin (µm) were measured from images using i-Solution Lite software. Otoliths were assigned a categorical margin code (one = 0% translucent area, two = 33%, three = 66%, four = 99%) based on the percentage of outer margin width relative to the width of the last fully-formed annuli, where a margin code of one indicates opaque ring formation (VanderKooy 2009). I conducted MIA using aggregated data from age-one otolith samples collected by the Mississippi Department of Marine Resources (2007, 2009 to 2013, n = 233) and age-one otolith samples collected for this study (2014 to 2015, n = 165) to increase sample size. The proportion of annuli formed (i.e., measured outer margin width as a proportion of the measured first annuli width) was examined as a function of capture month to estimate the timing of annuli deposition. A "biological" age estimate was then assigned:

$$\text{Age} = \frac{[(\text{annuli count} \cdot 365) + (\text{month} - 1) \cdot 30]}{365},$$

using the annuli count and month of capture, assuming January 1 as the birth date and April 1 as the annuli formation date (Nieland et al. 2002). The age estimate was adjusted based on the margin code, where individuals captured before or during April with a margin code of 3 or 4 were advanced by one year and individuals captured after April with a margin code of 1 or 2 were reduced by one year.

A multiple linear regression analysis was used to examine factors responsible for variability in otolith growth. The dependent variable in the model was proportion of annuli formed in age-one otoliths collected during 2007 to 2014 by the Mississippi Department of Marine Resources (2007, 2010 to 2013, n = 57) and collected for this

study (2014, n = 41). These data were collected during September to November of each year and were selected because representative samples were available across years for each month. Candidate models were evaluated using a stepwise forward selection approach with AIC comparison (Burnham and Anderson 2002). The independent variables evaluated were mean monthly sea surface temperature (SST, °C), year of capture, otolith radius (µm), and month of capture. Mean monthly SST data at a four km^2 spatial resolution were obtained from MODerate resolution Imaging Spectroradiometer (MODIS) sensors (www.oceancolor.gsfc.nasa.gov). A time series (January 2007 to December 2014) of the mean monthly SST value was constructed by averaging SST values across the study region, which was defined using a polygon covering the entire Mississippi Sound area (Figure 2). The continuous predictor variables, otolith radius and mean monthly SST, were scaled using two standard deviations and centered to the mean (Gelman 2008). I also examined the pairwise Pearson's product moment correlation between all predictor variables to remove highly correlated independent variables. The data were tested for normality with a Shapiro-Wilk test and for homogeneity of variance with a Bartlett's test. If the assumptions of a parametric test were met, an ANOVA test was used to determine if predictor variables had a significant effect on proportion of annuli formed.

The length-at-age relationship of female Southern Flounder was described using multiple non-linear models. A three-parameter VBGF (von Bertalanffy 1938) was used to estimate length-at-age:

$$L_t = L_\infty(1 - e^{-k(t-t_0)}),$$

where t represents age (y), L_t is the length (mm, TL) at a given age, L_∞ is the mean hypothetical maximum length (mm, TL), k is the growth coefficient (y^{-1}), and t_0 is the theoretical age at length of zero (y). Other candidate models used to describe length-at-age, including the two-parameter VBGF, Gompertz growth model, and logistic model, were also fit to the data. The two-parameter VBGF is:

$$L_t = L_\infty(1 - e^{-kt}).$$

The Gompertz growth model (Gompertz 1825) is:

$$L_t = L_\infty \, e(-\frac{1}{k} \, e^{-k(t-\frac{1}{k} ln\lambda)}),$$

where λ is the theoretical initial relative growth rate at age zero (y^{-1}) and k is the rate of exponential decrease of the relative growth rate with age (y^{-1}). The logistic length-at-age model (Ricker 1975) used is:

$$L_t = \frac{1}{L_\infty(1+e^{-k(t-t_i)})},$$

where k is a relative growth rate parameter (y^{-1}) and t_i corresponds to the age where the growth rate is at a maximum. These candidate length-at-age models were evaluated for goodness-of-fit and parsimony using Akaike information criterion (AIC). AIC values were compared to determine the best-fit model, indicated by the lowest AIC value. The ΔAIC and Akaike weight (ω_i) were calculated for model comparison and to evaluate relative model support. The 95% confidence intervals were calculated for each mean parameter estimate and used to compare results to published mean parameter estimates. Published mean values that were within the 95% confidence interval range of the mean parameter estimates reported in this study were interpreted as not significantly different.

The weight-at-length relationship was modeled using a power function:

$$W = aL^b,$$

where W is wet weight (g), L represents TL (mm), *a* is a coefficient term and *b* is an exponent describing change in length relative to weight. The 95% confidence intervals were calculated for each mean parameter estimate. There was insufficient data to describe a male-specific weight-at-length relationship for Southern Flounder, so only female-specific data were used.

The relative condition of individuals and temporal changes in relative condition were evaluated using a variation of Fulton's condition factor. Relative condition (K_{rel}) (Le Cren 1951), was calculated based on the relationship between observed wet weight (W) and expected mean weight predicted by the female weight-at-length relationship (W_{exp}):

$$K_{\mathrm{rel}} = \frac{\mathrm{W}}{\mathrm{W}_{\mathrm{exp}}}.$$

A relative condition value of one indicates perfect agreement between the observed weight and the expected mean weight predicted by the weight-at-length model. The significance level for all analyses was 0.05. All analyses were conducted using R 3.1.1 (R Core Team 2015).

Results

From September 2014 to February 2016, 522 Southern Flounder specimens (436 female, 52 male, 34 unsexed) were collected using various sampling methods. Fish were collected during all months of sampling, although sample sizes were limited during December, January, and March (Table 1). The majority (84%) of fish were captured within Mississippi waters, but others were collected from offshore Louisiana and Texas using trawls. A total of 204 fish were collected with gigs, 157 were collected with hook and line, 106 were collected with trawls, 23 were collected with gill nets, 13 were

collected by bow fishing, nine were collected with crab traps, eight were collected with seines, and two were collected with cast nets.

I processed a subsample of 367 otoliths for age estimation ranging in age from zero to four-plus years, and measured 398 age-one otoliths for marginal increment analysis. Using a resampling procedure, I observed that the coefficient of variation decreased as the sample size was increased from 50 to 350 (Figure 3), which shows that an increase in sample size decreases the variance of mean parameter estimates exponentially. The coefficient of variation was stabilized by about 350 samples for both parameter estimates, indicating that a sufficient total number of samples were processed for age estimation. Age estimates were in agreement between readers with a percent agreement of 82% or greater for each age class (Table 2). Within age-one otoliths, there was high individual variability in annuli deposition. I observed decreasing median proportion values between March and July with the minimum proportion in May, indicating that annuli formation occurred during these months (Figure 4). However, I observed the greatest decrease in median proportion values between March and April, and thus estimated that annuli formation occurs in April. There was a strong seasonal trend in the proportion of annuli formed, suggesting that annuli formation occurs once per year in Southern Flounder otoliths.

A stepwise multiple linear regression analysis indicated that mean monthly SST and year of capture have a significant effect on proportion of annuli formed in Southern Flounder otoliths. After candidate models were evaluated with AIC using a stepwise forward selection procedure, both of the significant predictor variables improved the fit of the model, indicated by a decreased ΔAIC value and an increased ω_i (Table 3).

However, the increase in ω_i was marginal compared to the models including otolith radius and month of capture as predictor variables (Table 3). Because TL and otolith radius were highly correlated (radius = 5.49 TL + 616.95, $p << 0.001$, $R^2 = 0.74$), only otolith radius was included as a predictor variable (Figure 5). An ANOVA analysis indicated that year and mean monthly SST have a significant effect on proportion of annuli formed (Table 4). Inter-annual variation in annuli proportion was observed within data collected from September to November, with the lowest median proportion observed in 2010 (Figure 6). Mean monthly SST had a negative effect on proportion of annuli formed (Figure 7), indicated by the negative slope coefficient in the linear relationship between SST and proportion (proportion = -0.010 SST + 0.76, $p << 0.001$, $R^2 = 0.39$).

The four models used to describe the length-at-age relationship for female Southern Flounder were all similar in mean length-at-age predictions (Figure 8). Where TL data were unavailable, SL measurements were converted to TL using the following relationship: TL = 1.14 SL + 18.94 ($p < 0.001$, $R^2 = 0.97$). There were insufficient data to fit a male-specific model, and the three-parameter VBGF model was the best-supported model to describe female-specific length-at-age (Table 5). The Gompertz and logistic model had lower model support than the three-parameter VBGF, and the two-parameter VBGF was not well supported. Because the three-parameter VBGF was the best supported candidate model and the most commonly reported model in the Southern Flounder literature, I compared the mean parameter estimates from this model to those reported at other locations using 95% confidence intervals (Table 6). None of the female-specific published mean parameter estimates from the GOM were significantly different from those reported in this study. However, there were significant differences between

female-specific mean parameter estimates and estimates from the Atlantic population, with a significantly lower L_∞ and higher k reported in this study.

All available female-specific weight and TL data collected in this study were used to describe the weight-at-length relationship for female Southern Flounder. The female weight-at-length relationship was described by the power function parameters a = $2.82e10^{-6}$ (95% CI: $1.90e10^{-6}$ to $4.17e10^{-6}$) and b = 3.24 (95% CI: 3.17 to 3.30). There were insufficient data to fit a male-specific model, although there was dimorphism in observed TL ranges between male and female Southern Flounder and all fish greater than 352 mm TL were female (Figure 9).

There was a weak seasonal trend in relative condition observed with elevated median values in the fall months preceding the winter spawning season. Specifically, the median monthly relative condition was greater than one during September through November (Figure 10). Median monthly relative condition was less than one during December through March, and May through August.

Discussion

In this study, I present a description of Southern Flounder age and growth in the north-central GOM and report growth parameter estimates specific to the Mississippi stock. Annuli deposition in Southern Flounder otoliths is variable but occurs between March and July annually and is influenced by inter-annual temporal variability in the environment. Southern Flounder growth is sexually dimorphic, and males are not a major component of the recreational fishery. Female-specific length-at-age parameter estimates were not significantly different from those reported at other locations in the GOM. Finally, there was intra-annual variation in relative condition. The results of this study

will help to inform state-level management of Southern Flounder in the GOM by providing knowledge of individual growth dynamics from the Mississippi stock.

Results indicate an annual frequency of otolith annuli deposition and support the conclusions from previous studies on Southern Flounder age and growth that validated the frequency of annuli deposition (Stunz et al. 2000, Fischer and Thompson 2004). The marginal increment analysis technique is an appropriate age validation method for Southern Flounder because this species is fast growing and has relatively few year classes (Fischer and Thompson 2004). Marginal increment analysis is preferable for use in young, fast-growing fish because annuli width decreases with age, and there is greater subjectivity associated with discerning narrowly spaced annuli in older, slow-growing fish (Campana 2001). However, age estimation is inherently subject to uncertainty and aging error can have negative effects on age-structured model parameter estimates (Quinn and Deriso 1999, Eklund et al. 2000). Growth parameter estimates could be improved by accounting for aging error in length-at-age models (Cope and Punt 2007). I also used marginal increment analysis to estimate that annuli formation occurs in April, which reduced the uncertainty of assigning age estimates and cohorts within a wide period of annuli deposition. This approach did not account for temporal variability in annuli deposition and included data from age-one individuals collected across multiple years. Considering inter-annual variability in annuli deposition rates may be an additional approach to reduce aging error (Pilling et al. 2007).

My results indicate that temporal variation in annuli deposition is driven in part by variation in environmental processes. In this study, the significant factors affecting proportion of annuli formed in age-one Southern Flounder otoliths were year of capture

and mean monthly SST. Otolith growth rates are known to be influenced by multiple factors, including fish size (Neilson and Geen 1982, Jones 1992), metabolism and feeding activity (Moksness et al. 1995), temperature, salinity, and dissolved oxygen concentration (Campana 1984, Hales and Able 1995). Although multiple environmental factors are responsible for inter-annual variability (Jobling 2002), temperature is most often cited as a factor affecting individual growth and, consequently, deposition rates of otoliths in both cold-water (Mosegaard et al. 1988, Otterlei et al. 2002) and in warm-water species (Black et al. 2011). Short-term fluctuations in water temperature can be detected in daily growth increments using laboratory experimentation (Campana 1984, Neilson and Geen 1982, Mosegaard et al. 1988, Bestgen and Bundy 1998). Long-term studies on the effects of temperature on annual otolith growth using field-collected samples are rarely reported in the literature. In one example, SST was correlated with otolith increment width across multiple decades of data from Red Snapper *Lutjanus campechanus* and Gray Snapper *Lutjanus griseus* in the GOM (Black et al. 2011). My results also indicate inter-annual variability in otolith increment width and suggest that SST has a negative effect on the proportion of annuli formed in age-one otoliths. Similarly, Pilling et al. (2007) demonstrated that elevated sea surface temperatures had a significant negative effect on annuli deposition rate in adult Atlantic Cod *Gadus morhua* otoliths. Understanding the effects of temperature variation on otolith growth has become increasingly critical due to climate change, particularly in commercially- and recreationally-harvested species for which accurate aging methods are needed for effective management (Eklund et al. 2000, Black et al. 2011).

This study demonstrates that Southern Flounder exhibit sexual dimorphism. Given this species' known sexual dimorphism in growth (Wenner et al. 1990, Stunz et al. 2000, Fischer and Thompson 2004) and limited male-specific data, only female-specific models were used to describe the length-at-age and weight-at-length relationships. Although I cannot address male-specific growth, the TL range of males was much smaller than that of females. Therefore, the use of female-specific growth parameter estimates in the present study may lead to overestimation of production in stock assessment models. However, I also observed that the majority of fish collected were female and very few fish collected inshore of the Mississippi Sound barrier islands were male. Many of the samples collected in this study were collected recreationally and thus were constrained by a 12-inch minimum length limit in Mississippi waters (GSMFC 2015). The female-biased sampling suggests that the selectivity associated with gear used in this study precluded the capture of smaller males. Other studies have suggested that there may be spatial differences in the distributions of Southern Flounder sexes (Midway et al. 2015), but there is insufficient data from the north-central GOM to support this hypothesis.

The use of multiple models to describe the length-at-age relationship is a recent trend in the fisheries literature (Burnham and Anderson 2002, Katsanevakis 2006), and thus, I used a multi-model approach to provide robust estimates of Southern Flounder length-at-age parameters. Only one previous study compared multiple models in describing sex-specific Southern Flounder growth (Fischer and Thompson 2004). In this study, the three-parameter VBGF was well supported to describe this species' growth, which supports the widespread use of this model in previous research. Although the

three-parameter VBGF had the lowest ΔAIC value, the top three models were all well supported models with ΔAIC values less than four (Burnham and Anderson 2004). In contrast, the two-parameter VBGF received very little relative model support compared to the other candidate models evaluated in this study, likely due to the limited number of fish captured smaller than 200 mm TL. The lack of smaller individuals is one potential source of bias in length-at-age parameter estimates, and the parameter estimates from this study may not be appropriate for describing early growth of larvae and juveniles. The length-at-age relationship description would be improved with the addition of smaller individuals to better reflect early growth (Pardo et al. 2013). In future research, a more rigorous resampling procedure could be used to evaluate whether sample size was sufficient across all age- and size-classes to describe growth through ontogeny.

The parameter estimates reported in this study are comparable to those reported at other locations, and no significant differences in the mean length-at-age parameter estimates were observed within the GOM. Female-specific estimates of L_∞ and k were significantly different from those reported in the Atlantic, with a lower L_∞ and higher k mean parameter estimates in this study compared to those reported in South Carolina (Wenner et al. 1990). However, length-at-age parameter estimates are variable within the GOM, which may be caused by several factors. Midway et al. (2015) suggests that there are spatial differences in Southern Flounder growth due to adaptations to local environmental conditions. However, there are also differences in sampling among studies (e.g., variable sample sizes and age ranges, gear types). In this study, I employed multiple gear types to collect samples from different sized individuals. The approach of using multiple sampling gears is advantageous for reducing bias associated with the selectivity

of a single gear type and increasing the precision of length-at-age parameter estimates (Wilson et al. 2015). Southern Flounder is primarily harvested using recreational gear types, so the sampling design appropriately replicated recreational fishing pressure on the Mississippi stock.

This study is the first to examine temporal variation in the relative condition of Southern Flounder females. Condition is a metric used to evaluate fitness of an individual and to observe temporal changes in growth dynamics (Le Cren 1951, Froese 2006). Southern Flounder individual growth likely varies intra-annually due to feeding activity and energetic investment in reproduction during the winter spawning season (Reagan and Wingo 1985, Shepard 1986, Fischer 1995). Although median relative condition was elevated in the months preceding the Southern Flounder spawning season, only slight variations in relative condition were observed during this study. Similarly, condition remained constant in another flatfish species, the Dab *Limanda limanda*, throughout the spawning season (Htun-Han 1978). Given the morphometrics of flatfish with a relatively small body-cavity size compared to overall body size, these results suggest that condition may not be useful for detecting intra-annual variation in flatfish growth.

Overall, this study provides a comprehensive description of Southern Flounder growth in the north-central GOM. This study provides critical knowledge about the local Southern Flounder population since this species' growth was not previously described in the north-central GOM. A species' individual growth dynamics influence how the population responds to fishing and are thus considered in management of the fishery (Adams 1980). Therefore, I expect that these results will be useful for the management of the Southern Flounder fishery in Mississippi and the broader GOM.

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Appendices

Table 1

Monthly sample size summary table

Month	**Female *n***	**Male *n***	**Mean TL**	**TL Range**
January	7	0	372	324 to 420
February	35	6	283	190 to 457
March	17	0	350	306 to 400
April	30	0	343	281 to 382
May	58	0	398	165 to 520
June	70	4	369	108 to 522
July	33	2	375	149 to 540
August	35	1	396	193 to 597
September	43	4	364	128 to 502
October	49	17	352	77 to 552
November	39	14	351	75 to 576
December	20	4	321	150 to 442
Total	*436*	*52*		

Monthly sample size (*n*), mean total length (TL, mm), and range of TL values for female and male Southern Flounder collected in the Mississippi Sound from September 2014 to February 2016.

Table 2

Summary statistics for age estimation

Age (y)	*n*	PA	Mean TL	SD	CV	Expected TL
0	83	96	287	52.1	18.2	106
1	209	87	369	40.1	10.9	315
2	60	97	449	52.4	11.7	416
3	11	82	483	43.6	9.0	465
4	4	100	494	22.1	4.5	488

Summary statistics for female and male Southern Flounder age estimation analysis (n = 367), including number of samples (n), percent agreement between readers (PA, %), mean observed total length (TL, mm), standard deviation (SD, mm), coefficient of variation (CV), and expected TL (mm) based on the three-parameter von Bertalanffy growth function for each age class.

Table 3

Multiple linear regression candidate models

Candidate Model	**ΔAIC**	**ω_i**
Proportion ~ SST + Year	0	0.35
Proportion ~ SST + Year + Radius + Month	0.13	0.32
Proportion ~ SST + Year + Radius	0.14	0.32
Proportion ~ SST	7.49	0.01
Proportion ~ 1	16.23	> 0.01

Candidate models evaluated using a forward stepwise AIC comparison procedure to describe proportion of annuli formed, defined as the measured outer margin width (µm) as a proportion of the measured first annuli width (µm), in age-one Southern Flounder otoliths. The independent variables evaluated were mean monthly sea surface temperature (SST, °C), year of capture, otolith radius (µm), and month of capture. ΔAIC is a measure of model support relative to the best candidate model and AIC weight (ω_i) represents the relative weight of model support.

Table 4

Linear regression model statistics

Coefficients	**df**	**Sum Sq**	**Mean Sq**	**F-Value**	***p*-Value**	**β Estimate**	**β SE**
Year	5	0.26	0.05	3.87	< 0.01		
SST	1	0.15	0.15	11.06	< 0.01	-0.06	0.018
Residuals	91	1.23	0.01				

ANOVA statistics and parameter estimates for the linear regression model describing proportion of annuli formed, defined as the measured outer margin width (µm) as a proportion of the measured first annuli width (µm), in age-one Southern Flounder otoliths. The independent variables included in this model were year of capture and mean monthly sea surface temperature (SST, °C).

Table 5

Length-at-age model parameters

Model	Parameter	Mean Parameter Estimate	95% CI	ΔAIC	ω_i
Three-parameter VBGF	L_∞	513.70	483.64 to 564.78	0.00	0.62
	k	0.67	0.46 to 0.90		
	t_0	-0.50	-0.94 to -0.21		
Gompertz	L_∞	501.53	475.99 to 542.07	1.65	0.27
	k	0.87	0.63 to 1.14		
	λ	0.90	0.58 to 1.40		
Logistic	L_∞	493.63	468.18 to 519.01	3.39	0.11
	k	1.07	0.80 to 1.34		
	t_i	0.38	0.25 to 0.50		
Two-parameter VBGF	L_∞	473.38	459.57 to 488.28	15.13	0.00
	k	1.13	1.03 to 1.23		

Multiple models describing the length-at-age relationship for female Southern Flounder collected in Mississippi waters (n = 274). The mean model parameters are reported with 95% confidence intervals. ΔAIC is a measure of model support relative to the best candidate model and AIC weight (ω_i) represents the relative weight of model support. The parameter L_∞ is the mean hypothetical maximum TL (mm), k is the growth rate coefficient (y^{-1}), t_0 is a theoretical age-at-length zero in the three-parameter VBGF, λ is the theoretical initial relative growth rate at age zero (y^{-1}) in the Gompertz model, and t_i corresponds to the age where the growth rate is at a maximum in the logistic model.

Table 6

Past research length at age parameter estimates

Study	**Location**	**Sex**	***n***	**L_∞ (mm)**	***k***	**t_0**
Nall 1979	Florida	combined	153	1461	0.03	1.86
Frick 1988	Florida/Alabama	female	139	540	0.47	0.1
Wenner et al. 1990	South Carolina	female	708	759	0.23	-0.57
		male	573	518	0.25	-1.07
Stunz et al. 2000	Texas	female	718	483	0.75	-0.31
		male	144	384	0.5	-1.38
Fischer and Thompson 2004	Louisiana	female	1128	556	0.51	-0.62
		male	137	332	1.03	-0.25
this study	Mississippi	female	274	513.70	0.67	-0.50
95% confidence intervals				483.64 to 564.78	0.46 to 0.90	-0.94 to -0.21

Summary of reported sample size (*n*) and length-at-age mean parameter estimates for Southern Flounder collected in Atlantic and Gulf of Mexico waters. In the three-parameter von Bertalanffy growth function, L_∞ is the mean hypothetical maximum TL (mm), *k* is the growth rate coefficient (y^{-1}), and t_0 is a theoretical age-at-length zero. The mean parameter estimates and 95% confidence intervals reported in this study are indicated in bold for comparison.

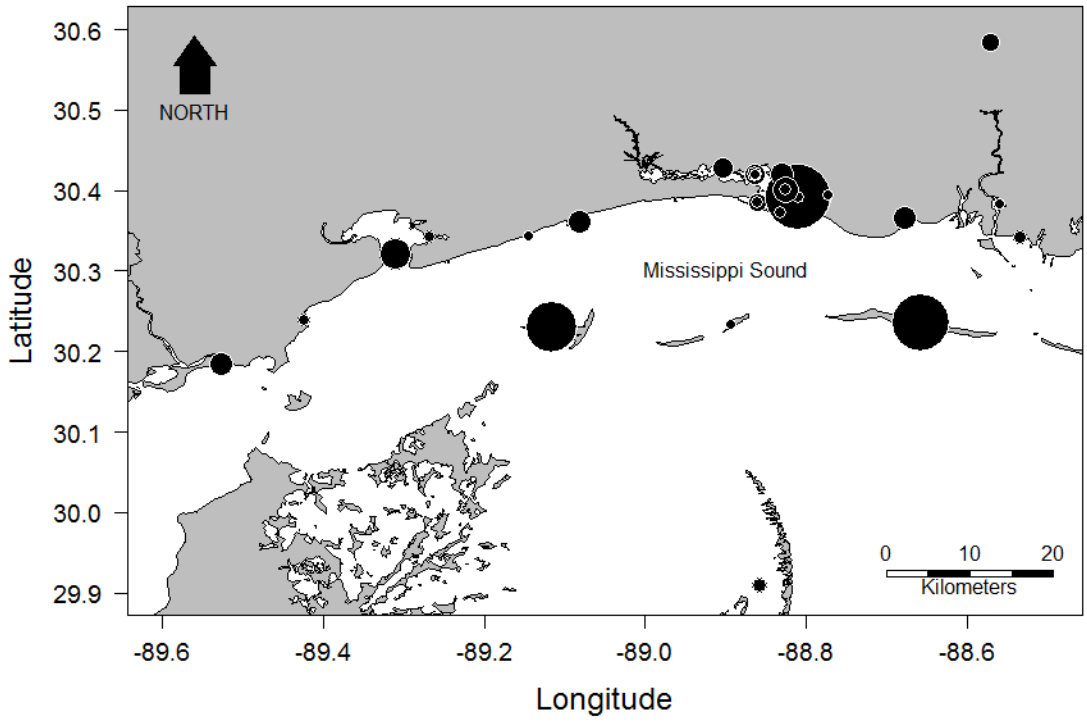

Figure 1. Map of Mississippi sampling area

Map of locations where Southern Flounder (*n* = 440) were collected using various gear types between September 2014 and February 2016. Size of the circles represents the relative magnitude of samples collected at each location.

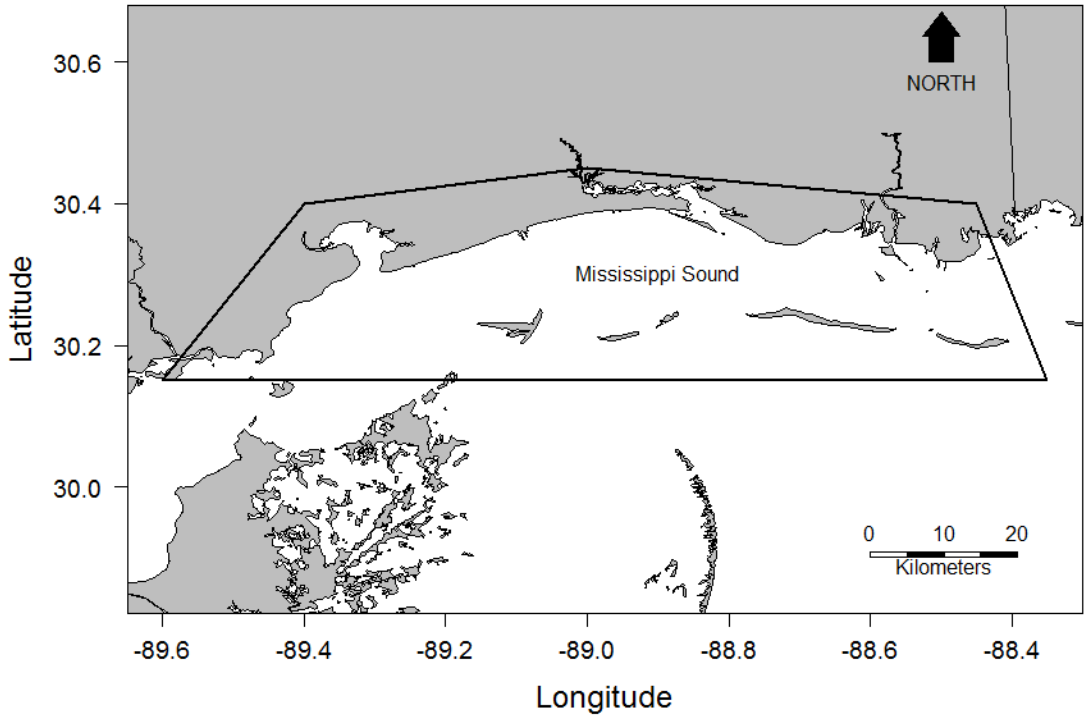

Figure 2. Map of temperature data study region

Polygon of the defined study region for which mean monthly sea surface temperature (SST, °C) data were extracted at a four km^2 spatial resolution from MODerate resolution Imaging Spectroradiometer (MODIS) sensors.

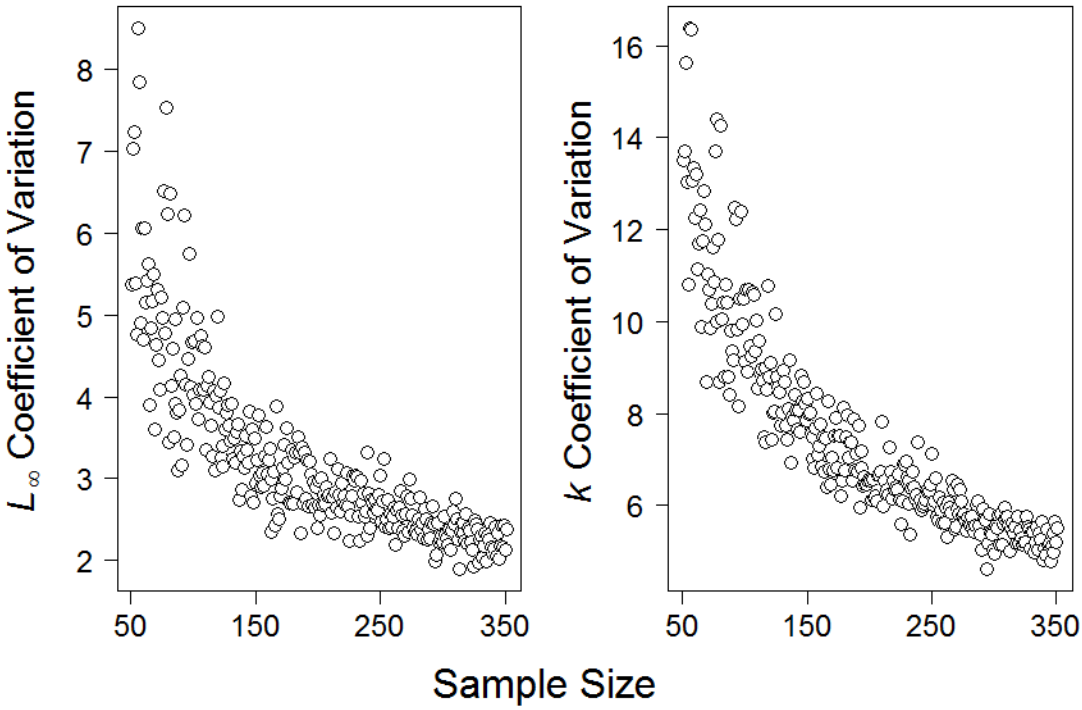

Figure 3. Sufficient sample size analysis

Coefficient of variation for the mean parameter estimates of L∞ and k in the two-parameter von Bertalanffy growth function as a function of increasing sample size. The parameter L_∞ is the mean hypothetical maximum TL (mm) and k is the growth rate coefficient (y^{-1}).

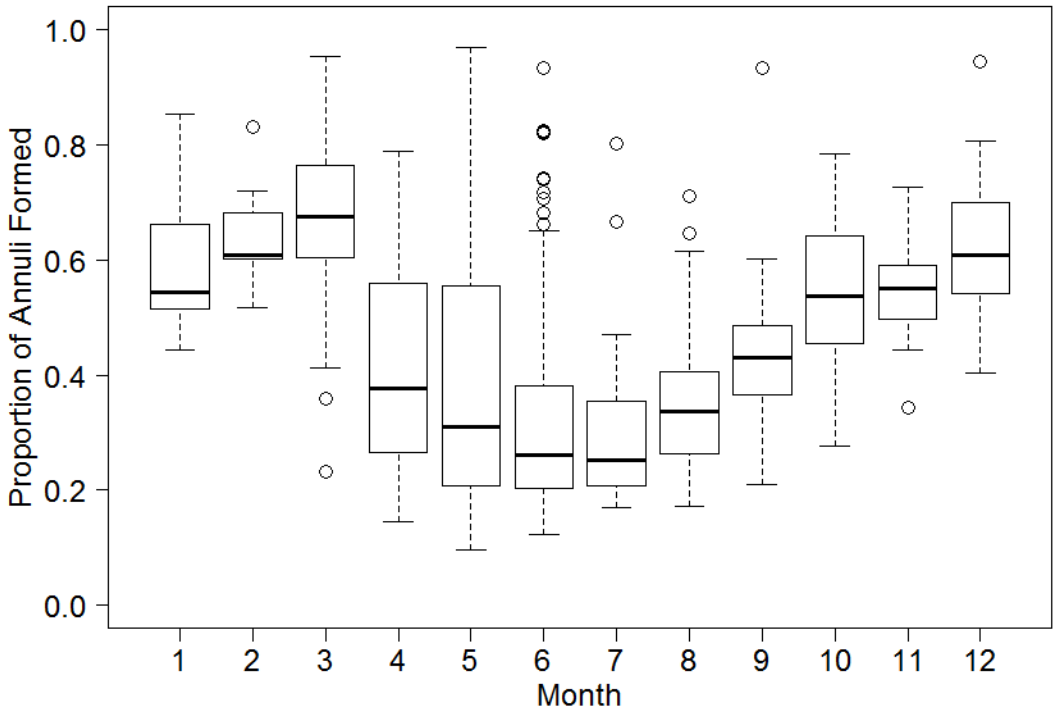

Figure 4. Marginal increment analysis

Boxplot of monthly measured marginal increment widths (µm) as a proportion of the last formed annuli from age-one otoliths (*n* = 398) collected during January (1) to December (12) from 2007 to 2016. Data were aggregated from otolith samples collected by the Mississippi Department of Marine Resources (2007, 2009 to 2013) and in this study (2014 to 2016). Dark bands indicate the median proportion, box edges indicate the 25% and 75% quartiles, and open circles indicate outliers in the data.

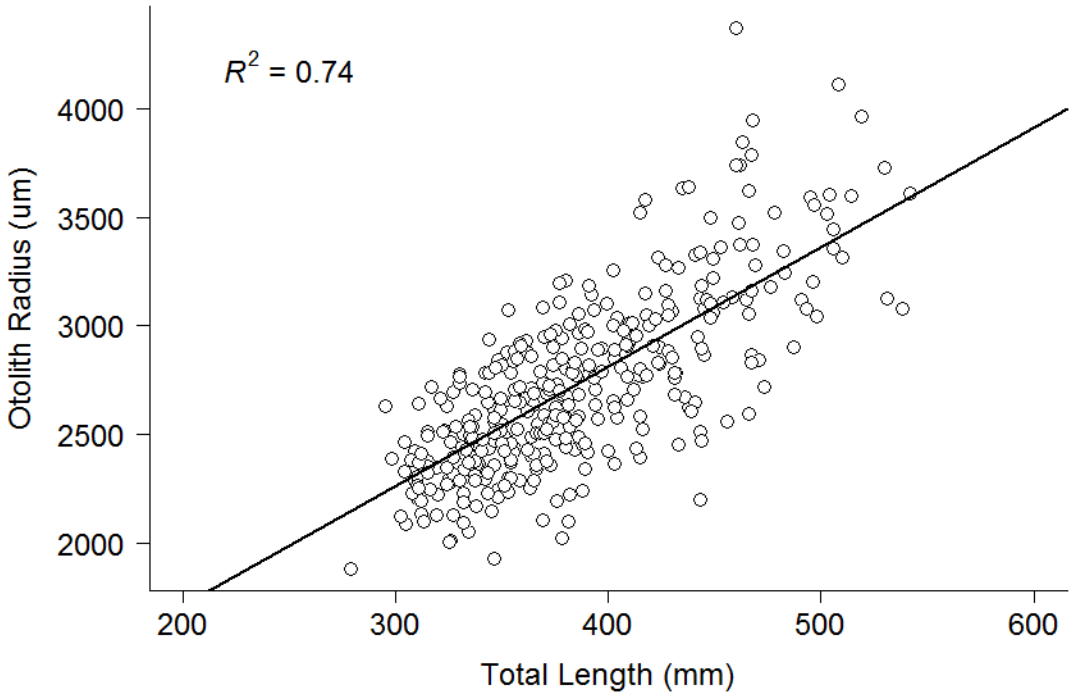

Figure 5. Otolith size linear regression

Linear regression between Southern Flounder total length (TL, mm) and otolith radius (µm) described by the relationship otolith radius = 5.49 TL + 616.95 ($p << 0.001$, $R^2 = 0.74$).

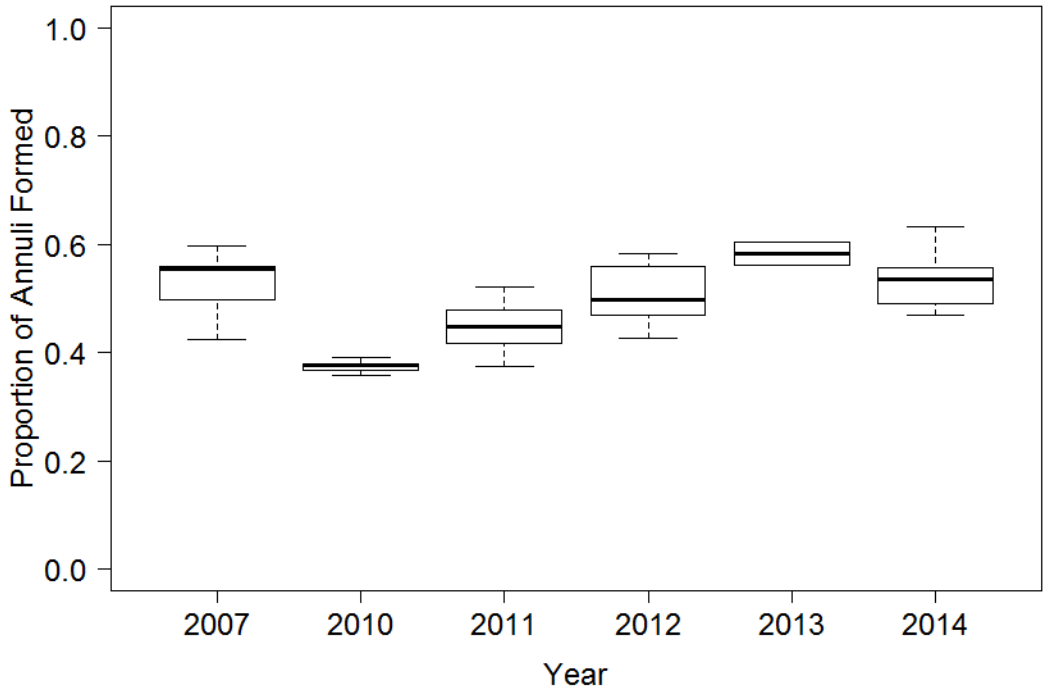

Figure 6. Inter-annual variation in annuli proportion

Boxplot of inter-annual variation in proportion of annuli formed, defined as the measured outer margin width (µm) as a proportion of the measured first annuli width (µm), in age-one otoliths (n = 98) collected during January (1) to December (12) from 2007 to 2014. Data were aggregated from otolith samples collected by the Mississippi Department of Marine Resources (2007, 2010 to 2013) and in this study (2014). Dark bands indicate the median proportion, box edges indicate the 25% and 75% quartiles, and open circles indicate outliers in the data.

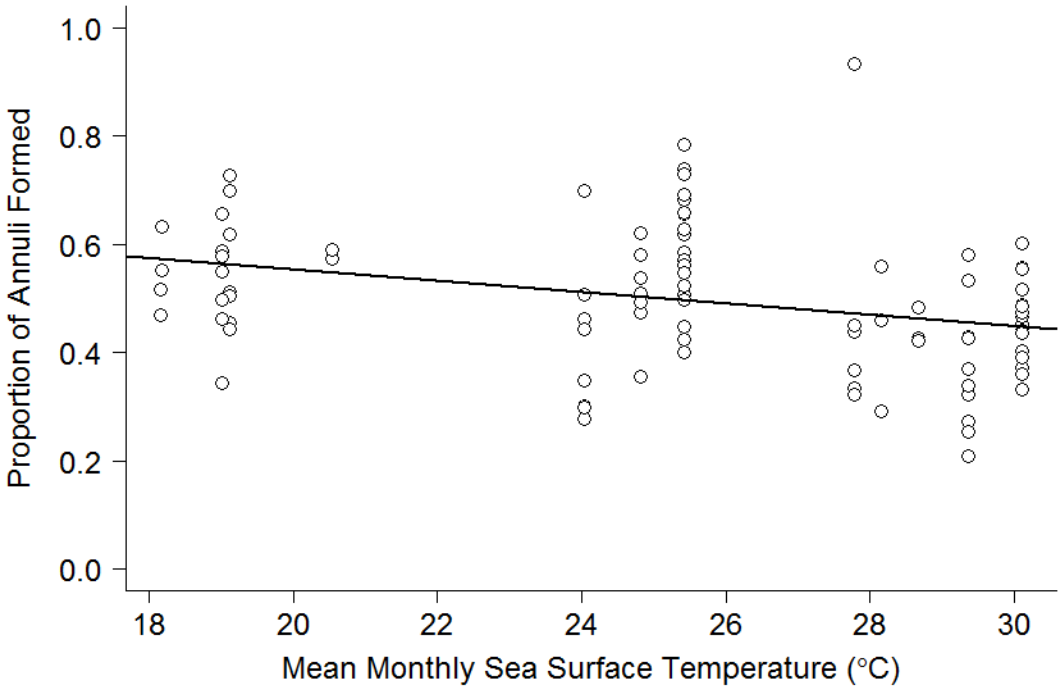

Figure 7. Linear regression temperature effect

Linear regression describing proportion of annuli formed, defined as the measured outer margin width (µm) as a proportion of the measured first annuli width (µm), as a function of mean monthly sea surface temperature (SST, °C) The line is described by the relationship proportion = - 0.010 SST + 0.76 ($p << 0.001$, $R^2 = 0.39$).

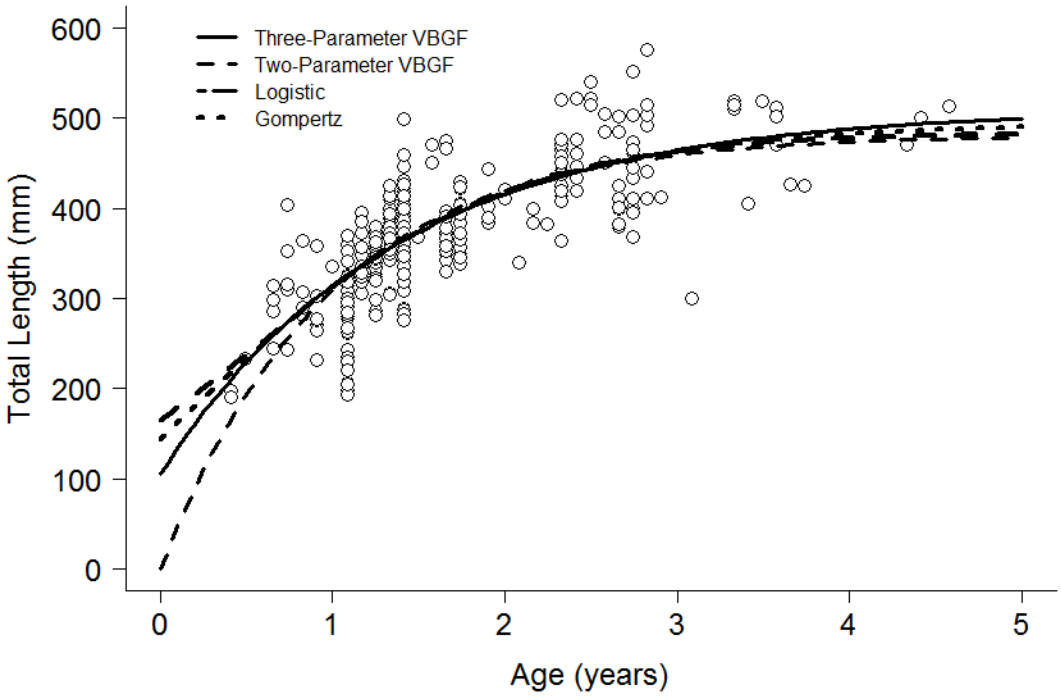

Figure 8. Length-at-age relationship multiple models

Multiple models describing the length-at-age relationship for female Southern Flounder (n = 274), including the three-parameter von Bertalanffy growth function (VBGF), two-parameter VBGF, logistic model, and Gompertz growth model. Models were fit to total length (mm) and adjusted age estimate (y) data.

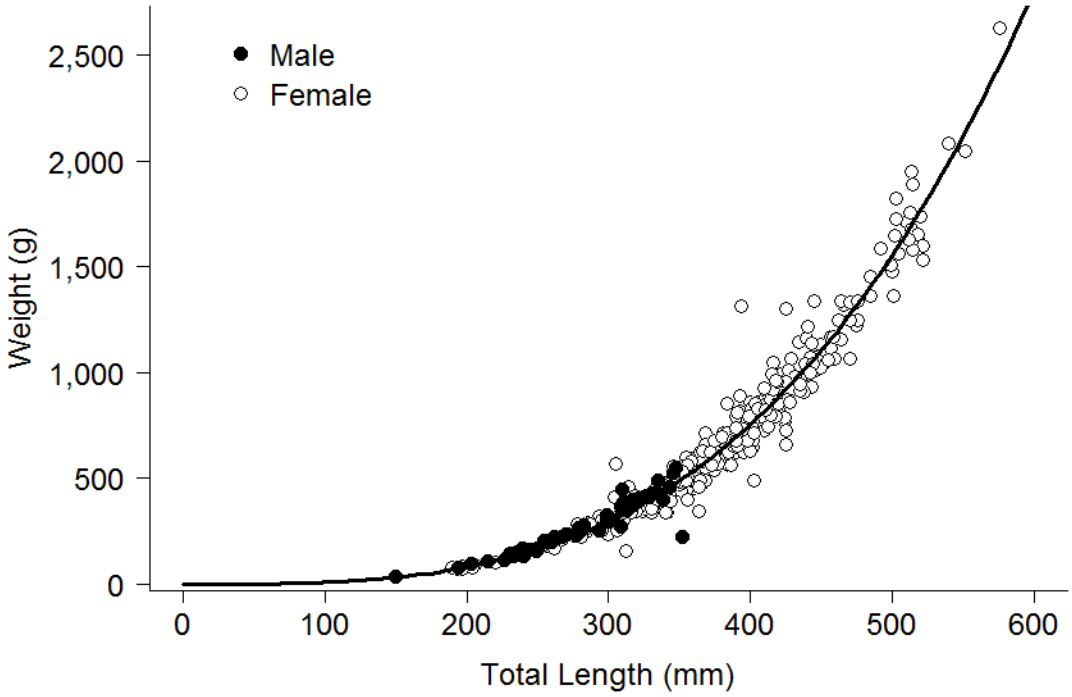

Figure 9. Weight-at-length relationship

The weight-at-length relationship for female (*n* = 436) Southern Flounder (open circles), where the line is a power function fit to female-specific data. There was insufficient data to fit a model for male (*n* = 52) Southern Flounder (closed circles).

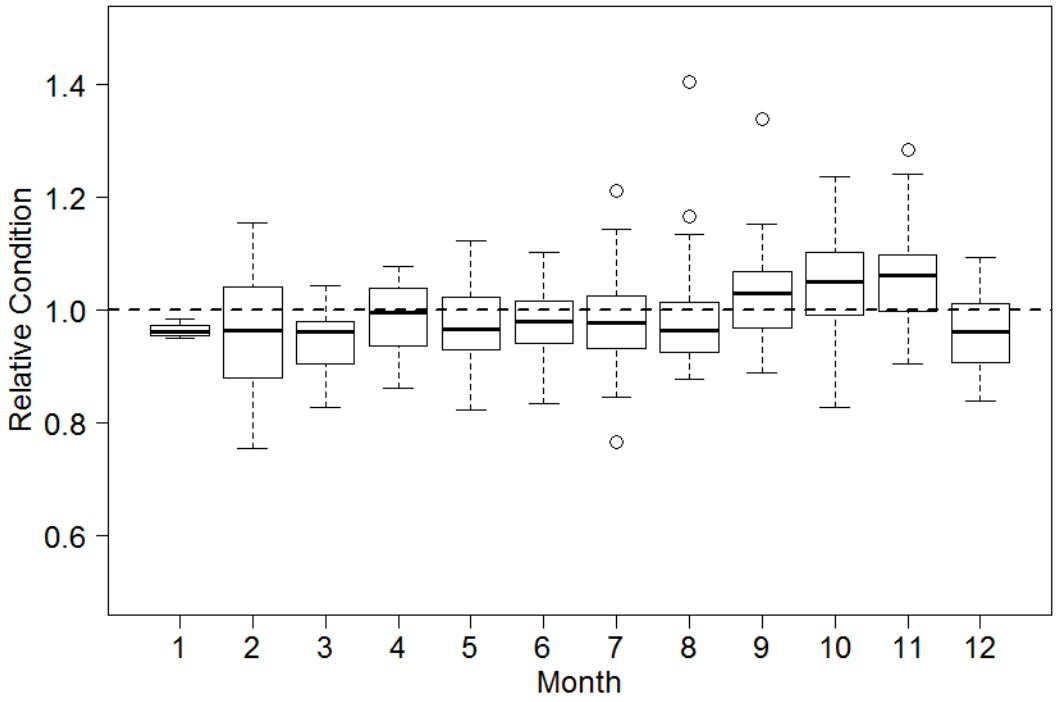

Figure 10. Relative condition variation

Boxplot of monthly variation in relative condition factor (*K*rel) for sexually mature females (*n* = 277) collected during January (1) to December (12). *K*rel is defined as the relationship between observed weight and expected mean weight predicted by the weight-at-length relationship (indicated by the dotted horizontal line at *K*rel = 1). Dark bands indicate the median *K*rel, box edges indicate the 25% and 75% quartiles, and open circles indicate outliers in the data.

CHAPTER III – REPRODUCTIVE BIOLOGY OF SOUTHERN FLOUNDER

Introduction

Southern Flounder *Paralichthys lethostigma* is a flatfish species in the family Paralichthyidae distributed throughout the Gulf of Mexico (GOM) (Hensley and Ahlstrom 1984). Southern Flounder are known to migrate to offshore continental shelf waters in the winter to spawn (Bailey et al. 2005), but an understanding of this species' reproductive biology is uncertain in the GOM. Specifically, Southern Flounder maturation and gonadal development are poorly understood in the north-central GOM. In fisheries science, an understanding of reproductive biology is essential because reproduction greatly influences fish population dynamics and the resilience of stocks (Beverton and Holt 1957, Lowerre-Barbieri et al. 2011a). Length- and age-at-maturity are particularly critical to describe because these estimates are used in stock assessment models (O'Brien et al. 1993, Murawski et al. 2001). Thus, having knowledge of reproductive biology is necessary to make informed management decisions based on estimates of spawning stock biomass.

There are few estimates of length-at-maturity and age-at-maturity for Southern Flounder reported in the GOM, and results from previous studies in the Atlantic indicate that Southern Flounder reach sexual maturity rapidly. Estimates of length-at-50% maturity (L_{50}) indicate that females in the Atlantic Southern Flounder population are mature at a total length (TL) between 345 and 408 mm (Monaghan and Armstrong 2000, Midway and Scharf 2012). All reported age-at-50%-maturity estimates are between one and two years (Stokes 1977, Monaghan and Armstrong 2000, Midway and Scharf 2012). Southern Flounder in Mississippi first reach sexual maturity around 230 mm TL and all

were mature by 340 mm TL (Etzold and Christmas 1979). Southern Flounder exhibit sexual dimorphism in length-at-maturity with greater length-at-first maturity and length-at-100% maturity observed in females than in males (Wenner et al. 1990). However, previously reported estimates of Southern Flounder maturity in Mississippi were not sex-specific and did not estimate the L_{50} parameter (Etzold and Christmas 1979), which is a parameter used to define maturity in stock assessment models (Trippel 1995, Lowerre-Barbieri et al. 2011a).

Southern Flounder spawning occurs in the winter season following an offshore migration (Stokes 1977, Benson 1982). The Southern Flounder winter spawning season has been described using the gonadosomatic index (GSI) and histological indicators. Gonad weight can be used as an indicator of spawning preparedness (Htun-Han 1978), so monthly GSI values are often used to describe annual reproductive development. For Southern Flounder collected in Louisiana, GSI values were elevated from August through November and declined in December, which indicate that increasing gonadal development occurred prior to spawning in December (Shepard 1986). Shepard (1986) recorded GSI from May to December, and this limited period of observation does not fully describe the annual trends in maturation for Southern Flounder. In another study, Fischer (1995) used both GSI and ovarian histology to determine that the Southern Flounder spawning season lasts about 60 days and occurs from December through January in Louisiana.

Southern Flounder reproductive strategy has not been recently described in the GOM. Batch spawning was observed in laboratory-reared Southern Flounder, and each female spawned more than three times throughout the spawning season duration (Arnold

et al. 1977). However, spawning behavior in a laboratory setting likely does not reflect spawning in a natural population (Conover and Kynard 1984). The presence of different oocyte stages throughout the spawning season was indicative of batch spawning in Southern Flounder collected from Louisiana waters (Fischer 1995). This is the only known example of batch spawning documented in wild-caught Southern Flounder. A description of Southern Flounder spawning dynamics is needed because the frequency of spawning affects lifetime fecundity, and consequently, population dynamics (Lowerre-Barbieri et al. 1998).

The purpose of this research is to describe the reproductive biology of Southern Flounder in the north-central GOM. Therefore, the following objectives were developed: 1) to estimate the mean length-at-50% maturity and age-at-50% maturity for female Southern Flounder based on histological phase classifications; 2) to estimate the duration of the spawning season using mean GSI values and histological indicators; and 3) to describe the spawning dynamics and gonadal development of female and male Southern Flounder using histological analysis. The knowledge gained from this research will improve understanding of Southern Flounder life history and management of the GOM stock.

Methods

Southern Flounder were collected in the north-central GOM from September 2014 to February 2016. Hook and line fishing, gigging, and trawling were the most common sampling methods used. Sampling occurred at multiple locations primarily within the Mississippi Sound (Figure 1), but samples from fish caught in other Gulf states and offshore locations were also included (Figure 9). Additional samples were obtained from

local fishing tournaments and from fishery-independent research surveys. Fish were immediately placed on ice following collection and processed in the laboratory within 24 hours to preserve gonad tissues.

The sex of each fish was determined by macroscopic examination of gonads and gonadal tissue was preserved for histological analysis. Each specimen was measured for TL (mm) and total weight (g). Whole gonads were removed, weighed to the nearest 0.01 g, and evaluated macroscopically for reproductive phase (Table 7 & 8). A cross section no larger than 1 cm^3 from the middle of one gonad was placed into a histology cassette and fixed in 10% neutral buffered formalin for at least one week. A 1:20 volumetric ratio of tissue to formalin was used to ensure adequate penetration and preservation of the gonadal tissue.

Gonadal tissue samples were processed using standard histological techniques. The sample cassettes were rinsed overnight with low-flowing tap water to prepare for dehydration of the gonad samples and embedding in paraffin. After rinsing, samples were placed in 60% ethanol for two hours, drained, placed in 70% ethanol for two hours, drained, and replaced in 70% ethanol for a minimum of two hours. Next, the preserved gonad samples were dehydrated using various dilutions of ethanol up to 100%, cleared using Shandon Xylene substitute, and impregnated with Paraplast Plus in a Shandon Excelsior Tissue Processor (Table A1). All steps were performed under vacuum to maximize the penetration of reagents into the tissues. Tissues were embedded within one hour of cycle completion using a Shandon Histocentre 2 Embedding Center. To embed tissues, a small amount of Paraplast was placed in the bottom of a stainless-steel mold and the gonad tissue was positioned in a manner to obtain the best cross-section. The

tissue was secured by briefly cooling the paraffin, and the cassette base was placed on top of the mold. The mold was then completely filled with Paraplast. The cooled Paraplast and tissue block were removed from the mold and the excess paraffin trimmed off. To prepare for tissue sectioning, an S/P Brand Tissue Flotation Bath was filled with distilled water. One cap-full of Surgipath STAY ON, a tissue section adhesive, was added and the bath heated to 37-42°C. Prior to sectioning, the blocks were placed on ice. Blocks were sectioned at a thickness of 4 µm using an AO Rotary Microtome with a disposable Accu-Edge Low Profile Microtome Blade. Sections were placed in the water bath and the best two from each specimen floated onto a slide. Each slide was labeled and placed on a slide warmer for a minimum of two hours to completely dry. The staining process included removing the paraffin, rehydrating the sample, staining the various tissue components, and then dehydrating the section in a sequence of solutions with varying soak times (Table A2). Slides were stained following a regressive method of hematoxylin staining (Luna 1968) using Hematoxylin 2 and counterstained with Eosin Y (Richard-Allan Scientific). Solution baths were rotated or discarded and replaced as needed. Slides were cover-slipped using a mounting medium (Richard-Allan Scientific) and allowed to dry completely.

Tissue samples were examined from the anterior, middle, and posterior sections of both the left and right gonad in three spawning capable females to determine if oocyte development was homogenous throughout the gonad. The percent coverage of each stage present in individual tissue sections was determined from images taken using a Nikon Eclipse 50i compound microscope with DXM 1200C camera and ACT-1C software. The entire tissue section was imaged and three photos were randomly selected from each slide

for oocyte examination using an Image J software point grid. The number of grids covering each stage were then counted and divided by the total number of grid points, resulting in a percentage of total area for each stage (modified from Tomkiewicz et al. 2011). A Pearson's Chi-square test was used to identify differences in distribution of oocyte stages between the left and right ovaries, and among the anterior, middle, and posterior regions of the ovary (α level = 0.05). When significant differences were detected, a pairwise comparison was made using multiple Chi-square tests with a Bonferroni adjusted critical value (α = 0.05 / number of tests).

Individuals were assigned to a developmental phase following the reproductive phase classification described by Brown-Peterson et al. 2011 (Table 7 & 8) and coded as immature (0) or mature (1). Females were classified as sexually mature when fish enter the developing phase and cortical alveoli oocytes are observed (Brown-Peterson et al. 1988, Brown-Peterson et al. 2011, Lowerre-Barbieri 2011b). Non-reproductively active females in the regenerating phase were identified using histological indicators (Table 7) and classified as mature. Mean length-at-50% maturity was estimated for female Southern Flounder (n = 332) using a two-parameter logistic model:

$$M_{TL} = \frac{1}{1+e^{-r(\mathrm{TL}-L_{50})}},$$

where r is the instantaneous rate of change and L_{50} is the TL-at-50% maturity. Age-at-maturity was back calculated using the length-at-age relationship of female Southern Flounder reported in Chapter II. The 95% confidence intervals of the mean parameter estimates were also calculated and reported. The significance level was 0.05. All analyses were conducted using R 3.1.1 (R Core Team 2015).

The spawning season duration was estimated using a combination of GSI data and histological examination of gonadal development. The GSI value was calculated for each individual using the following equation:

$$\mathrm{GSI} = \left(\frac{\mathrm{GW}}{\mathrm{GFBW}}\right) \cdot 100 \ ,$$

where GW is the gonad weight (g) and GFBW is the gonad-free body weight of the fish (g). A linear regression of GSI and GFBW was conducted to confirm that GSI is an indicator of reproductive development independent of body size (Jons and Miranda 1997). Mean monthly GSI values were calculated for sexually mature individuals and reported with standard error. For histological analysis, the percent coverage of each stage present in individual tissue sections was determined from images taken at 10x magnification for females and 40x magnification for males, and analyzed using Image J software as described above. Gonadal development of males was further described using histological assessment of the spermatogenic maturity index (SMI). The SMI method involves estimation of the area fractions of various tissue categories characterized by progressive spermatogenic development stages in histological sections of the testes (Tomkiewicz et al. 2011). The mean SMI was calculated for each male using the following equation:

$$\mathrm{SMI} = 0.0\mathrm{F}_{\mathrm{Ts}} + 0.4\mathrm{F}_{\mathrm{Sg}} + 0.6\mathrm{F}_{\mathrm{Sc}} + 0.8\mathrm{F}_{\mathrm{St}} + 1.0\mathrm{F}_{\mathrm{Sz}} \ ,$$

where F is the frequency of occurrence for the indicated cell type (Ts = testicular somatic cells, Sg = spermatogonia, Sc = spermatocytes, St = spermatids, Sz = spermatozoa). The index weighs the volume fractions of the different tissues (somatic cells and germ cell

stages) and describes testis development on a scale of 0 to 1. Males were classified as sexually mature when fish enter the developing phase, and primary spermatocytes are observed (Brown-Peterson et al. 2011). Mean monthly SMI values were calculated for mature individuals and reported with standard error.

Results

Samples from 369 Southern Flounder (332 females, 37 males) were collected from September 2014 to February 2016. A total of 142 fish were collected with gigs, 126 were collected with hook and line, 70 were collected with trawls, 19 were collected with gill nets, six were collected with seines, and six were collected with crab traps. Of these fish, 58 were collected from locations outside the Mississippi Sound (Figure 11).

The homogeneity of oocyte development between and within ovaries was assessed to support the sampling protocol used in this study. There were no significant differences in female oocyte stage distribution between the left and right ovaries (χ^2 = 7.19, $p > 0.05$), indicating that oocyte distribution was homogenous. However, there were significant differences in oocyte stage distribution among the anterior, middle, and posterior regions of the ovaries ($\chi^2 = 26.78$, $p = 0.0083$). To identify which regions were homogenous in oocyte stage distribution, I used three Chi-square tests to make pairwise comparisons between regions and compared *p*-values to a Bonferroni adjusted critical value ($\alpha = 0.017$). There were no significant differences between the anterior and posterior regions ($\chi^2 = 15.31$, $p = 0.018$) or between the middle and posterior regions ($\chi^2 = 2.72$, $p = 0.84$), but significant differences were detected between the middle and anterior regions ($\chi^2 = 19.491$, $p = 0.0034$). Thus, sampling from the mid-posterior region of the ovary was an appropriate method used in this study.

The smallest mature female Southern Flounder collected was 245 mm TL and all were mature by 368 mm TL. There were insufficient data to estimate L_{50} for males, but the smallest mature male collected was 150 mm TL and all were mature by 335 mm TL. Based on the two-parameter logistic function (Figure 12), the *r* mean parameter estimate was 0.0412 mm^{-1} (95% CI: 0.033 to 0.053 mm^{-1}) and the L_{50} mean parameter estimate was 303.80 mm TL (95% CI: 295.53 to 310.82 mm TL) for females. The age-at-50%-maturity was estimated as one year by back calculation using the three-parameter von Bertalanffy growth function mean parameter estimates reported in Chapter II. The mean parameter estimate of L_{50} was significantly higher than L_{50} estimates reported in Louisiana and significantly lower than L_{50} estimates reported in the Atlantic (Table 9).

Spawning seasonality of Southern Flounder was described initially using GSI and SMI data. There was a weak linear relationship observed between GSI and GFBW (R^2 = 0.09, $p < 0.001$) in sexually mature females. In reproductively active mature females, the linear relationship between GSI and GFBW, although significant, explained little of the variance in GSI ($R^2 = 0.06$, $p = 0.04$). Female mean GSI values remained constant during January to September and were elevated in October to December (Figure 13). The highest mean GSI value was observed in November, and several individuals had elevated mean GSI values in December. Results from a one-factor ANOVA test indicated monthly differences in mean GSI for females, and mean GSI in November was significantly higher than mean GSI values in all other months (post hoc Tukey HSD test: $p < 0.05$). Increasing male GSI values were observed in September to October, and the highest mean GSI value was observed in November (Figure 14). Male GSI remained elevated from October to December and in February. Similarly, increasing SMI values were

observed in September to December, and the highest mean SMI value was observed in December (Figure 15). The results of these analyses indicate that spawning likely occurs from November to January and ceases in February.

The Southern Flounder spawning season duration was further defined using histological classification of reproductive phase. Five reproductive phases were observed in both females and males, including the early developing subphase in females and males, and the early-, mid-, and late- germinal epithelium (GE) subphases in spawning capable males. However, no actively spawning individuals were collected for either sex. The percent agreement between macroscopic and histological phase classification was 39% for females and 29% for males. The developing phase was most accurately identified by macroscopic examination with 94% agreement. In contrast, the immature and regenerating phases were poorly classified by macroscopic examination with 23% and 26% agreement, respectively. Immature and regenerating females were observed throughout the year. The greatest percentages of early developing and developing females were observed in October, indicating the beginning of the reproductive season (Table 10). Spawning capable females were most frequently observed in November and regressing females were observed in January and February. Immature and regenerating males were observed throughout all months in which collection occurred. The greatest percentages of early developing and developing males were observed in September and October, respectively (Table 11). Spawning capable males were observed from October through December, and regressing males were observed in February. The histological results support the November through January spawning season indicated by GSI and SMI data.

Each reproductive phase was described histologically for female Southern Flounder. Female fish were classified as immature when 100% of oocytes were in the primary growth stage (Table 12), and primary growth oocytes were tightly packed with interstitial tissue and a thin ovarian wall (Figure 16). Female fish were also classified as regenerating when 100% of oocytes were in the primary growth stage, but the presence of various sized perinucleolar primary growth oocytes as well as reduced interstitial tissue indicated the regenerating phase rather than the immature phase (Figure 17). Primary growth and cortical aveolar oocytes were most abundant in early developing females, with only 3.97% of oocytes in the primary vitellogenic stage (Table 12, Figure 18). Compared to early developing females, a greater mean percentage of all vitellogenic oocyte stages were observed in developing females (Figure 19). The majority of oocytes were in secondary and tertiary vitellogenic stages for spawning capable females (Table 12, Figure 20). Because various stages of oocytes were observed simultaneously within an individual ovary, Southern Flounder can be classified as batch spawners with asynchronous oocyte development. Regressing females were identified by the presence of atresia and post-ovulatory follicle complexes, indicative of recent spawning (Figure 21).

The reproductive development of male Southern Flounder was described histologically. Male fish were classified as immature based on the presence of 100% primary spermatogonia (Table 13) and the absence of lumens in the testicular tissue (Figure 22). The regenerating phase was distinguished from the immature phase in males by the presence of empty lumens with spermatogonial proliferation near the periphery of the tissue (Figure 23) and the presence of residual spermatozoa (Table 13). The majority of tissue was spermatogonia in early developing males, and all spermatogenic stages

were present in developing males (Table 13, Figure 24). Spawning capable males were identified by the presence of spermatozoa in the lumens (Figure 25 & 26). The early-, mid-, and late-GE subphases were differentiated by increasing percentages of spermatozoa (Table 13) as well as an increasing number of lobules with discontinuous GE. Specifically, the presence of continuous GE throughout lobules was used to identify early GE subphase males (Figure 25), and discontinuous GE throughout lobules was used to identify late GE subphase males (Figure 26). Regressing males were characterized by reduced spermatogenesis with residual spermatozoa present (Figure 27).

Discussion

This research provides a description of Southern Flounder reproduction in the north-central GOM. The results of this study indicate that Southern Flounder mature rapidly and mean estimates of L_{50} were significantly different from previous estimates reported for this species. Histological evidence and GSI were used to demonstrate that the Southern Flounder spawning season duration is from November to January. Results from histological analysis suggest that Southern Flounder exhibit a batch spawning strategy with asynchronous oocyte development. Finally, the gonadal development of males and females is described in detail using histological indicators. The information reported in this study will greatly improve understanding of Southern Flounder life history and management of the GOM stock.

Female Southern Flounder in the north-central GOM reach maturity between 245 and 368 mm TL and within one to two years. The current estimate of L_{50} was significantly higher than previously described in the GOM (Fischer 1995), although L_{50} in this study is about equal to the current 12-inch (305 mm) minimum length limit in

Mississippi (GSMFC 2015). The description of maturity reported by Fischer (1995) indicates that female Southern Flounder in Louisiana grew rapidly to 50% maturity by 229 mm TL but all females were mature by 509 mm TL. Estimates of female Southern Flounder L_{50} from this study were significantly lower than previously reported estimates in the Atlantic. Differences in L_{50} are likely due to different population dynamics between the GOM and Atlantic stocks (Midway et al. 2015) and indicate that Southern Flounder in the GOM are faster to reach maturity. Maturity is a fundamental life-history trait that varies in response to population-level influences (Adams 1980, Shuter 1990). Age- and size-at-maturity of temperate flatfish species are influenced by multiple factors, such as environmental variation and fishing pressure (Roff 1982). For example, decreases in age-at-maturity have been related to increasing exploitation rates in multiple commercially harvested flatfish stocks, including American Plaice *Hippoglossoides platessoides* in Grand Banks (Trippel 1995). Thus, variation in Southern Flounder L_{50} estimates could be related to spatial differences in environment conditions and historic levels of fishing mortality. Female age-at-50% maturity was estimated as one year in this study, which is in agreement with results reported in the Atlantic (Monaghan and Armstrong 2000, Midway and Scharf 2012). However, observations by Monaghan and Armstrong (2000) demonstrated that 73.5% of age-one fish were mature but age-one females did not exhibit increasing GSI before the spawning season. Although age-one females are able to grow to maturity, age-two females may be more likely to migrate offshore during the spawning season (Stokes 1977).

The use of histology in this study provides a detailed assessment of Southern Flounder maturation and gonadal development. Previous studies in the GOM have not

used histological phase classification to estimate Southern Flounder maturity (Stokes 1977, Etzold and Christmas 1979), with the exception of Fischer (1995). Histological classification of maturity is preferable to macroscopic classification because defining characteristics of reproductive phases can be clearly identified (Hunter and Macewicz 1985, West 1990, Lowerre-Barbieri et al. 2011a). The misidentification of reproductive phases can have implications for estimation of biological reference points and, consequently, for management (King and McFarlane 2003). For example, the estimates of L_{50} increased by 33 mm TL for the Southern Flounder stock in North Carolina when using histological phase assignment methods compared to macroscopic phase assignment (Midway and Scharf 2012). Increasing L_{50} from the previously reported L_{50} value using histological phase assignment caused a 10% decrease in predicted spawning potential ratio. In this study, the percent agreements between macroscopic and histological classification were low for both sexes, indicating the value of using histology to categorize reproductive development (West 1990). Although macroscopic classification of reproductive phase is commonly used as a rapid assessment method, Midway and Scharf (2012) demonstrate that the resulting error in maturity estimates can contribute to shifts in biological reference points from spawning stock biomass per recruit models.

Several results from this study indicate that Southern Flounder spawn from November to January in the north-central GOM. First, mean GSI values were elevated during the months preceding November and mean SMI remained elevated through February in males. These results are in agreement with previous studies, which also observed peak GSI values in November and December (Shepard 1986, Fischer 1995). The mean GSI values reported in this study are lower in magnitude, but the trend of

increasing GSI leading up to November is similar to trends reported in past studies. Gonadal development is expected to increase prior to the spawning season and decrease as spawning activity diminishes (West 1990), although GSI may remain elevated in indeterminate batch spawning fish species. The use of GSI may be biased when comparing samples from different sized fish, but GSI should be independent of body size to reflect reproductive development (Le Cren 1951, West 1990). In Southern Flounder there was a weak linear relationship observed between GSI and GFBW that explained 9% of the variance in GSI. Further, for reproductively active mature females GFBW explained 6% of the variance in GSI, which supports the use of GSI data as an indicator of reproductive development in this study. Second, histological indicators were used to identify reproductive phase throughout annual reproductive development. Specifically, spawning capable individuals were observed in November indicating spawning preparedness, and regressing individuals were observed in February indicating the end of the spawning season. Results from this study not only support the conclusions from previous studies that reported a December to January spawning season in the GOM (Shepard 1986, Fischer 1995), but also indicate that spawning activity begins as early as November and may continue into February. The absence of actively spawning individuals collected in the winter is a limitation to the conclusions from these data. Therefore, the duration of the spawning season was inferred based on the presence of spawning capable individuals in November and regressing individuals in February.

Batch spawning and asynchronous oocyte development in Southern Flounder was supported by the presence of oocytes in various developmental stages within female ovaries (Wallace and Selman 1981, Lowerre-Barbieri et al. 2011b). This result supports

the conclusions of studies focused on both laboratory-spawned (Arnold et al. 1977) and natural spawning Southern Flounder (Fischer 1995). Batch spawning is common in other flatfish species, including the North Sea Dab *Limanda limanda* (Htun-Han 1978), Dover Sole *Microstomus pacificus* (Hunter et al. 1992), Tasmanian Greenback Flounder *Rhombosolea tapirina* (Barnett and Pankhurst 1999), and Summer Flounder *Paralichthys dentatus,* in the Middle Atlantic Bight (Morse 1981). A multiple spawning strategy, with asynchronous development of oocytes throughout the spawning season, may represent an adaptation to maximize reproductive potential (Murua and Saborido-Rey 2003). Southern Flounder is a warm-water species, so an extended spawning season with multiple spawning events could increase lifetime fecundity (Morse 1981). The documentation of batch spawning in Southern Flounder is useful because batch fecundity estimates inform annual fecundity estimates, which are used in stock assessment models of spawning stock biomass (McEvoy and McEvoy 1992, Goodyear 1993).

Based on the analysis of data collected in this study, female and male Southern Flounder exhibit dimorphic reproductive development patterns and distributions before the spawning season. Males were observed in the developing phase in September and in the spawning capable phase as early as October. In contrast, females were observed in the spawning capable phase starting in November. This difference in timing indicates that males may start developing earlier in the year and have a longer period in the spawning capable phase than females. Differential development patterns between sexes is expected because females have a relatively greater energetic investment in the production of offspring than males (Trivers 1972, Rijnsdorp and Witthames 2005). Sex-specific differences observed in this study may also be related to spatial distributions of males and

females. Because female Southern Flounder grow faster and reach larger sizes than males (Chapter II), females were more vulnerable to the primarily recreational sampling techniques used and were captured more frequently during all months of sampling. However, all spawning capable male Southern Flounder were captured at offshore locations and spawning capable females were collected only at inshore locations prior to offshore migration at the start of the spawning season. These results support the hypothesis that males migrate offshore earlier than females and may remain in offshore locations (Wenner et al. 1990, Midway and Scharf 2012).

In conclusion, this research provides a description of Southern Flounder reproduction, including a female-specific estimation of length- and age-at-maturity, estimation of the spawning season duration, identification of batch spawning strategy, and a histological description of male and female gonadal development. However, there are still outstanding research needs to understanding the reproductive biology of Southern Flounder. Collection of actively spawning females during the winter spawning season would allow for estimation of fecundity and spawning frequency, as well as classification of fecundity type. The management of stocks for resilience against disturbance is a critical goal of fisheries science, and thus, an understanding of how reproductive potential affects population productivity is valuable for effective management (Lowerre-Barbieri et al. 2011a). Because current research on Southern Flounder reproduction in the north-central GOM was lacking before this study, these results will be useful to inform future stock assessments for this species.

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Appendices

Table 7

Female reproductive phase terminology

Phase	Definition	Macroscopic Description	Histological Description
Immature	Never spawned.	Small ovaries, clear with no distinct blood vessels.	Contains only oogonia and primary growth oocytes, has a thin ovarian membrane and little space between oocytes with interstitial tissue present.
Developing	Gonads in preparation to spawn.	Enlarging ovaries, blood vessels more distinct.	Gonad can contain primary growth, cortical alveolar, and early and mid vitellogentic oocytes. Late vitellogenic oocytes rare. Some atresia possible but no postovulatory follicles.
Early Developing	Developing subphase.		Gonad composed only of primary growth and cortical alveolar oocytes. May have early vitellogentic oocytes.
Spawning Capable	Fish will spawn during the spawning season.	Large ovaries, blood vessels prominent.	Abundance of late vitellogenic oocytes present. Gonad may also contain primary growth, cortical alveolar, postovulatory follicles, and atresia of vitellogenic and/or hydrated oocytes. Early stages of oocyte maturation may be present.
Actively Spawning	Fish is spawning, has spawned within 12 hrs, or will spawn within 12 hrs.		Separated from spawning capable fish by evidence of widespread oocyte maturation indicated by lipid and/or yolk coalescence, germinal vesicle migration, and/or hydration of oocyte. Postovulatory follicles ≤12 hrs can be present.
Regressing	Fish will not spawn again this season.	Flaccid ovaries, blood vessels prominent.	Atresia at any/all stages present and abundant. Primary growth oocytes becoming more abundant with most vitellogenic oocytes undergoing atresia. Postovulatory follicles possible.
Regenerating	Mature fish not reproductively active.	Small ovaries, blood vessels reduced but present.	Gonad contains oogonia and primary growth oocytes (perinucleolar stage common) and has a thick ovarian wall with reduced interstitial tissue. May have atresia or muscle bundles present.

Female classification terminology adapted from Brown-Peterson et al. (2011).

Table 8

Male reproductive phase terminology

Phase	Definition	Macroscopic Description	Histological Description
Immature	Never spawned.	Small testes, often clear and threadlike.	Contains only primary spermatogonia, no lumen in lobules.
Developing	Gonads in preparation to spawn.	Enlarging testes, color becomes translucent.	Gonads may contain secondary spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa in spermatocysts. Spermatozoa not present in lumen of lobules or in sperm ducts. Germinal epithelium continuous.
Early Developing	Developing subphase.		Gonad composed only of primary spermatogonia, secondary spermatogonia, and primary spermatocytes.
Spawning Capable	Fish will spawn during the spawning season.	Large testes, translucent white in color.	Spermatozoa in lumen of lobules and/or sperm ducts. All stages of spermatogenesis present. Spermatocysts throughout testes, and active spermatogenesis. Germinal epithelium can be continuous or discontinuous.
Actively Spawning	Fish is spawning, has spawned within 12 hrs, or will spawn within 12 hrs.	Release of milt with gentle pressure on abdomen.	Macroscopic identification only.
Early GE		Histological only.	Continuous GE in all lobules throughout the testes.
Mid GE		Histological only.	Continuous GE in all lobules at testes periphery, discontinuous GE in lobules near ducts.
Late GE		Histological only.	Discontinuous GE in all lobules throughout the testes.
Regressing	Fish will not spawn again this season.	Flaccid testes reduced in size.	Residual spermatozoa present in lumen of lobules and in sperm ducts. Widely scattered spermatocysts near periphery containing secondary spermatocytes, spermatids, and spermatozoa. Spermatogonial regeneration of germinal epithelium in testes periphery.
Regenerating	Mature fish not reproductively active.	Small testes, translucent white in color.	No spermatocysts present. Lumen of lobule often nonexistent. Proliferation of spermatogonia and germinal epithelium continuous throughout. Residual spermatozoa present in lumen of lobules and in sperm ducts.

Male classification terminology adapted from Brown-Peterson et al. (2011).

Table 9

Past research maturity estimates

Study	**Location**	**Sex**	***n***	***Age*$_{50}$ (y)**	***L*$_{first}$ (mm)**	***L*$_{50}$ (mm)**	***L*$_{100}$ (mm)**
Stokes 1977	Texas	combined		2			
Etzold and Christmas 1979	Mississippi	combined		3	230		340
Wenner et al. 1990	South Carolina	female	377		320		380
		male	318		230		310
Fischer 1995	Louisiana	female			200	229	509
Monaghan and Armstrong 2000	North Carolina	female		1		345	
Midway and Scharf 2012	North Carolina	female	451	1		408	
this study	Mississippi	female	332	1	245	303.8	368
95% confidence intervals						295.53 to 310.82	

Summary of reported maturity estimates for Southern Flounder collected in Atlantic and Gulf of Mexico waters, including sample size (*n*), age-at-50% maturity (y), length-at-first maturity observed (L_{first}, mm), length-at-50% maturity (L_{50}, mm), and length-at-100% maturity (L_{100}, mm).

Table 10

Monthly reproductive phase summary for females

Month		**Immature**		**Developing**	**Spawning Capable**	**Regressing**	**Regenerating**
	n		***Early Developing***				
January	7	0	0	0	0	14.29	85.71
February	38	57.89	0	0	0	13.16	28.95
March	17	23.53	0	0	0	0	76.47
April	21	9.52	0	0	0	0	90.48
May	33	6.06	0	0	0	0	93.94
June	47	6.38	0	0	0	0	93.62
July	21	19.05	0	0	0	0	80.95
August	20	0	0	0	0	0	100
September	29	6.90	0	0	0	0	86.21
October	43	2.33	58.14	20.93	0	0	18.60
November	36	2.22	16.67	11.11	38.89	0	11.11
December	20	35.00	15.00	5.00	5.00	0	40.00
Total	*332*						

Monthly percentages of samples and sample size (*n*) for female Southern Flounder collected in the north-central Gulf of Mexico from September 2014 to February 2016.

Table 11

Monthly reproductive phase summary for males

Month		**Immature**		**Developing**	**Spawning Capable**			**Regressing**	**Regenerating**
	n		***Early Developing***		***EGE***	***MGE***	***LGE***		
January	0	0	0	0	0	0	0	0	0
February	4	0	0	0	0	0	0	50.00	50.00
March	0	0	0	0	0	0	0	0	0
April	0	0	0	0	0	0	0	0	0
May	0	0	0	0	0	0	0	0	0
June	3	100	0	0	0	0	0	0	0
July	2	0	0	0	0	0	0	0	100
August	1	100	0	0	0	0	0	0	0
September	4	0	50.00	0	0	0	0	0	50.00
October	9	11.11	0	33.33	33.33	22.22	0	0	0
November	10	30.00	0	0	20.00	20.00	10.00	0	20.00
December	4	25.00	0	25.00	0	25.00	25.00	0	0
Total	*37*								

Monthly percentages of samples and sample size (*n*) for male Southern Flounder collected in the north-central Gulf of Mexico from September 2014 to February 2016. Spawning capable males are classified by early- (EGE), mid- (MGE), and late- germinal epithelium (LGE) subphases.

Table 12

Reproductive phase summary by percent oocyte stage

Phase	***n***	**% PG**	**% CA**	**% Vtg1**	**% Vtg2**	**% Vtg3**	**% POF**	**% Atresia**
Immature	1	100	0	0	0	0	0	0
Developing	13	30.15	35.19	30.49	3.72	0.15	0	0.39
Early Developing	5	49.56	46.36	3.97	0	0	0	0.11
Spawning Capable	15	10.14	13.93	22.71	18.48	32.28	0	2.83
Regressing	3	73.16	6.32	0	0	0	6.58	13.95
Regenerating	2	100	0	0	0	0	0	0
Total	*39*							

Sample size (*n*) of individuals examined (three images per individual) and mean percentages of oocytes in each stage for reproductive phases observed in female Southern Flounder from the north-central Gulf of Mexico. Oocyte stages include primary growth (PG), cortical aveolar (CA), primary vitellogenic (Vtg1), secondary vitellogenic (Vtg2), tertiary vitellogenic (Vtg3), post-ovulatory follicle complex (POF), and atresia.

Table 13

Reproductive phase summary by spermatocyte stage

Phase	***n***	**% SG**	**% SC**	**% ST**	**% SZ**
Immature	5	100	0	0	0
Developing	3	19.51	56.40	20.43	3.66
Early Developing	2	66.50	32.52	0.97	0
Spawning Capable					
Early GE	5	7.17	43.41	30.81	18.60
Mid GE	6	5.72	28.25	34.14	31.89
Late GE	2	7.69	21.89	23.08	47.34
Regressing	4	14.13	0	0	85.87
Regenerating	6	75.09	18.05	0	3.25
Total	*33*				

Sample size (*n*) of individuals examined (three images per individual) and mean percentages of spermatocytes in each stage of spermatogenesis for reproductive phases observed in male Southern Flounder from the north-central Gulf of Mexico. Spermatogenic stages include primary and secondary spermatogonia (SG), primary and secondary spermatocytes (SC), spermatids (ST), and spermatozoa (SZ).

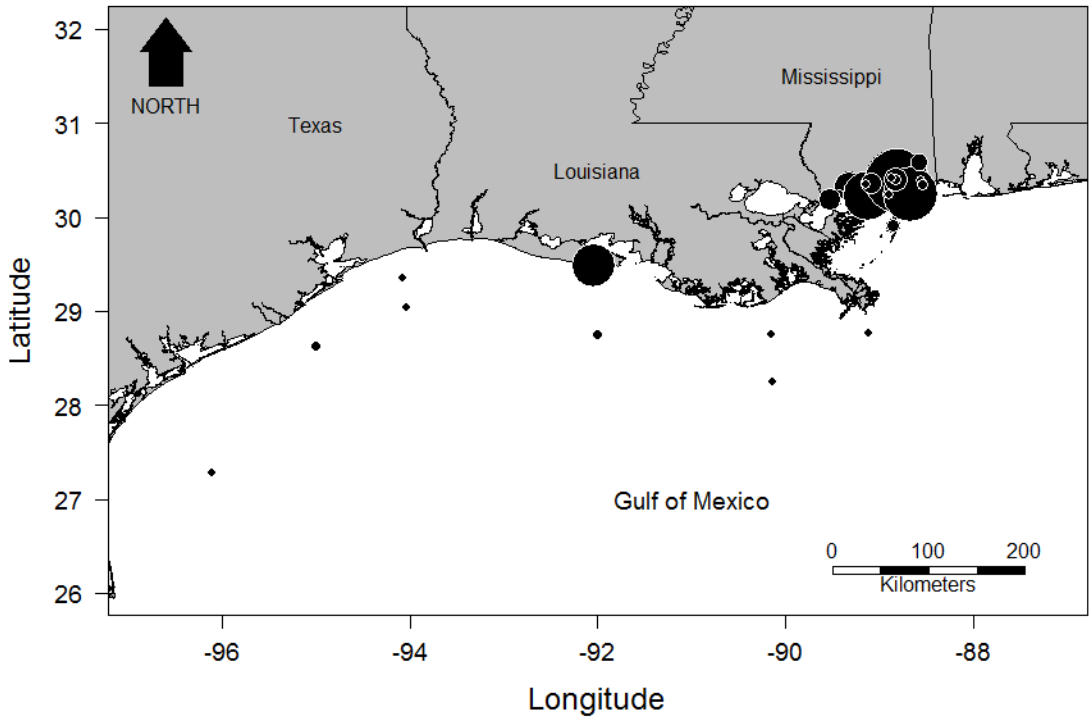

Figure 11. Map of Gulf of Mexico sampling area

Map of locations where Southern Flounder (*n* = 369) were collected using various gear types between September 2014 and February 2016. Size of the circles represents the relative magnitude of samples collected at each location. See Chapter II for a detailed map of Mississippi Sound sampling locations.

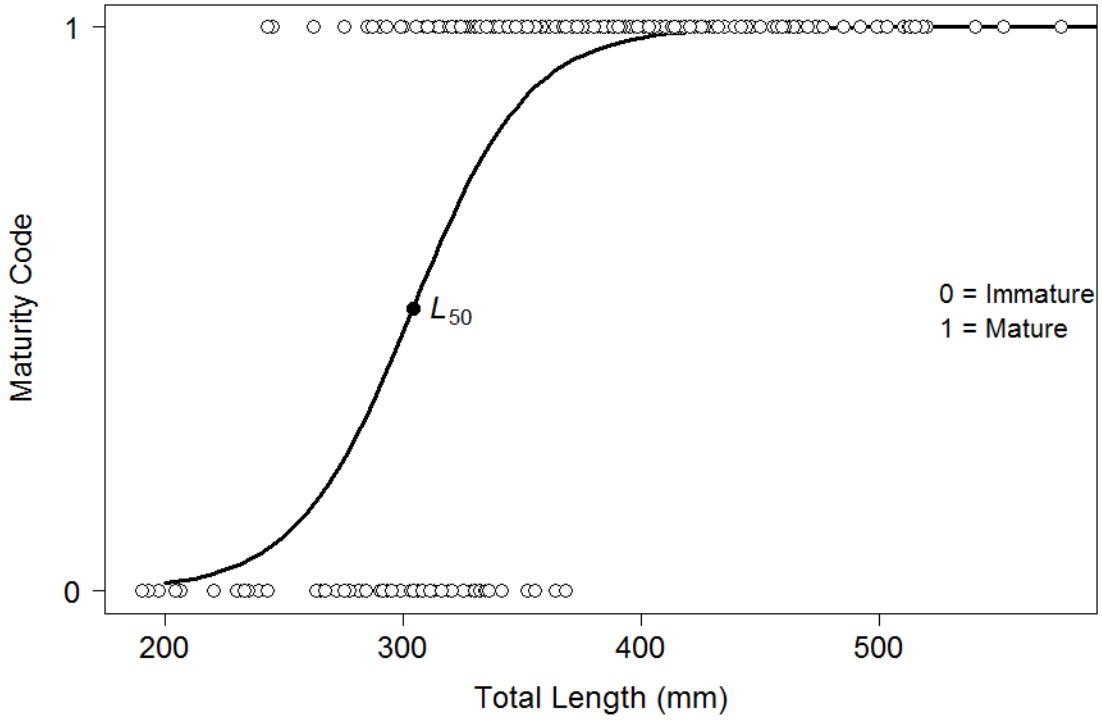

Figure 12. Length-at-maturity logistic model

Logistic model describing the length-at-maturity for female Southern Flounder (n = 332) from the north-central Gulf of Mexico, where L_{50} represents the mean parameter estimate for total length-at-50% maturity. Individuals were assigned a binomial maturity code indicating immature (0) or mature (1) status.

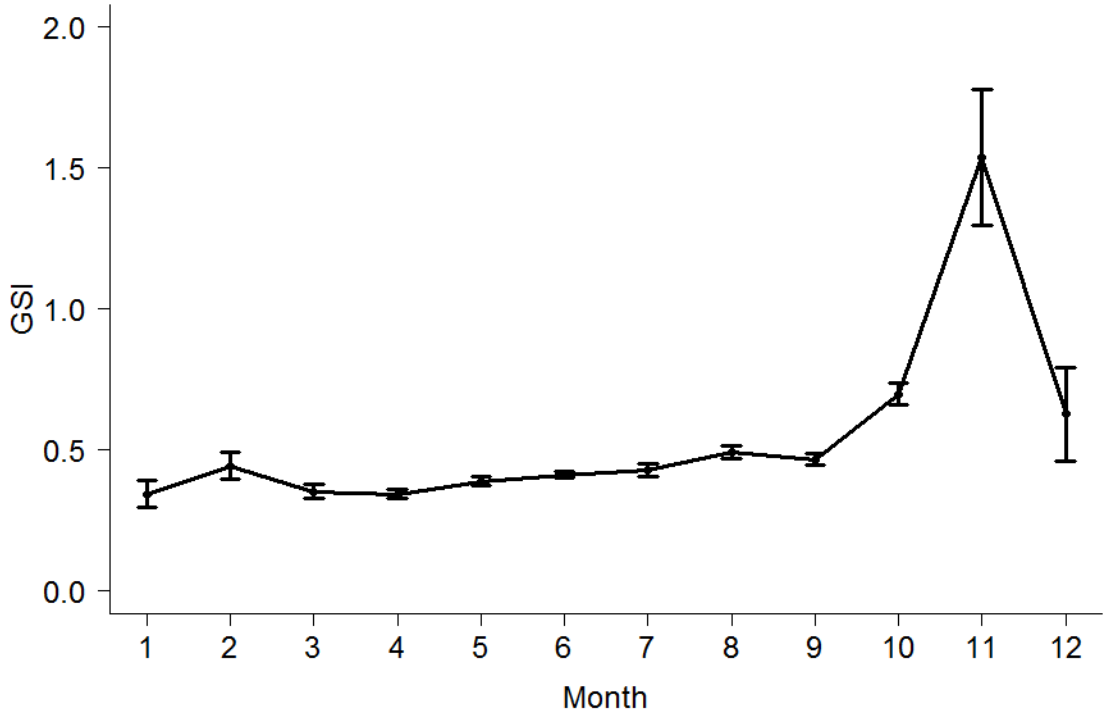

Figure 13. Monthly GSI for females

Mean monthly gonadosomatic index (GSI) for sexually mature female Southern Flounder (*n* = 277) collected during January (1) to December (12). Error bars indicate the standard error of the mean monthly GSI value.

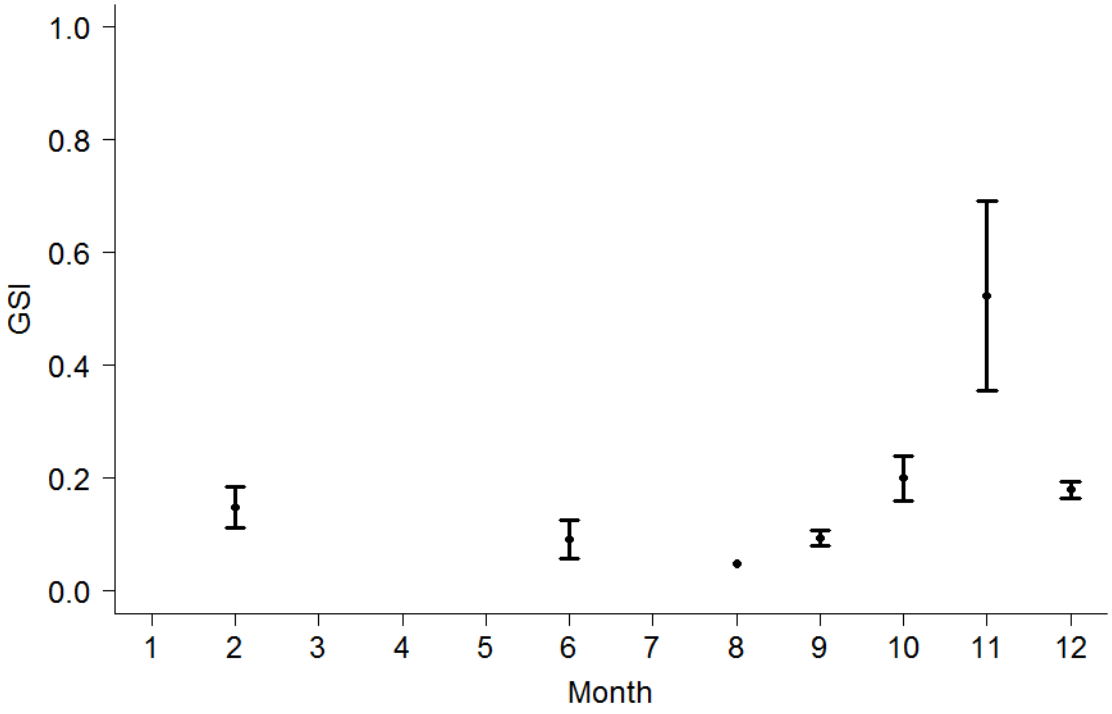

Figure 14. Monthly GSI for males

Mean monthly gonadosomatic index (GSI) for mature male Southern Flounder (*n* = 23) collected during January (1) to December (12). Error bars indicate the standard error of the mean monthly GSI value.

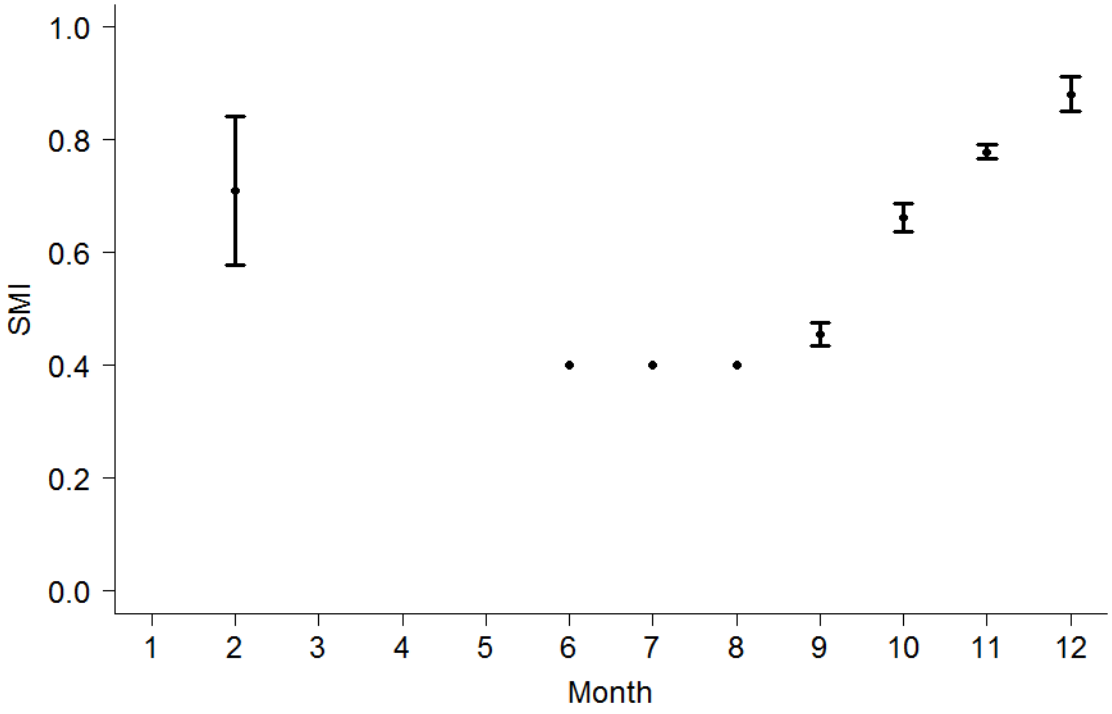

Figure 15. Monthly SMI for males

Monthly spermatogenic maturity index (SMI) for mature male Southern Flounder (n = 31) collected during January (1) to December (12). Error bars indicate the standard error of the mean monthly SMI value.

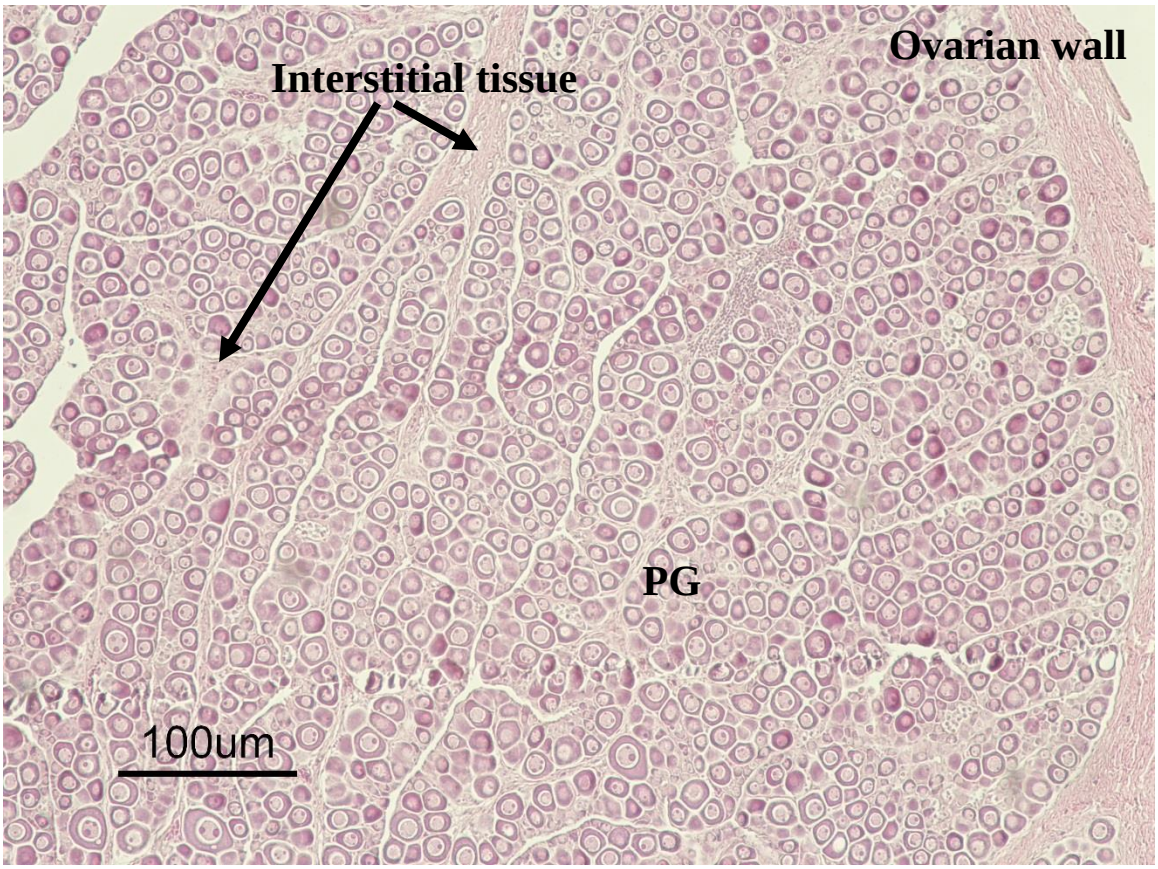

Figure 16. Immature female histology image

Histology sample imaged at 10x magnification from an immature female Southern Flounder (239 mm TL) caught in November 2014. Immature females were identified by the presence of small, tightly-packed primary growth oocytes (PG) with a thin ovarian wall and interstitial tissue.

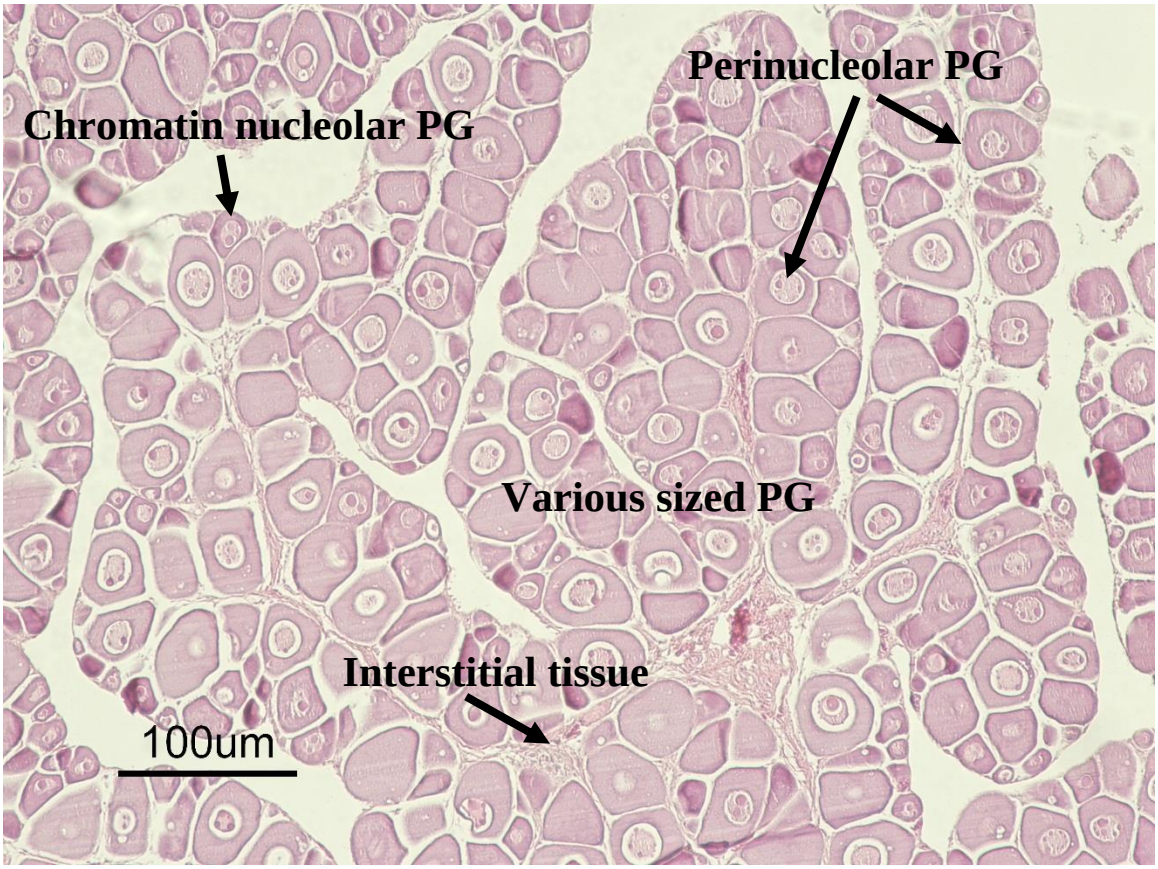

Figure 17. Regenerating female histology image

Histology sample imaged at 10x magnification from a regenerating female Southern Flounder caught in September 2014. Regenerating females were identified by the presence of primarily perinucleolar primary growth oocytes (PG) of various sizes. Chromatin nucleolar PG oocytes were also observed with reduced interstitial tissue compared to immature females.

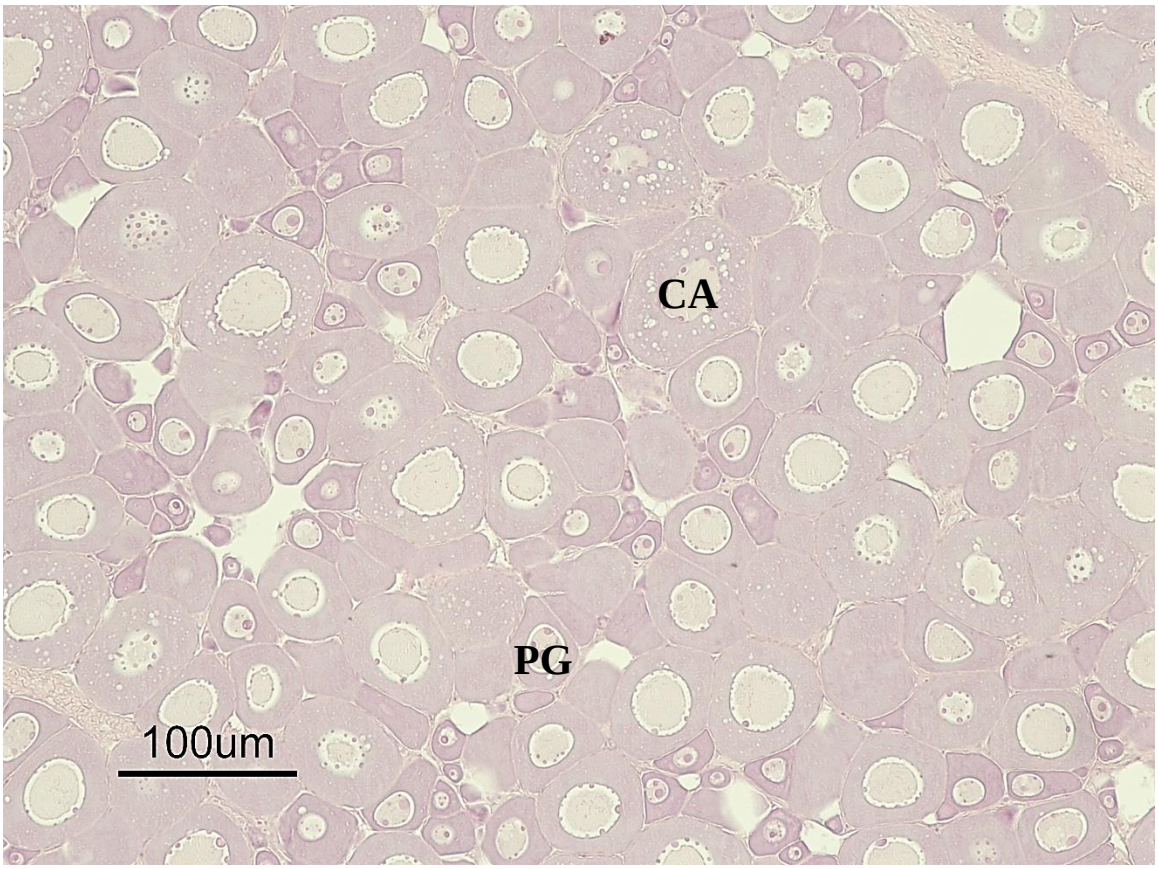

Figure 18. Early developing female histology image

Histology sample imaged at 10x magnification from an early developing female Southern Flounder caught in October 2014. Early developing females were identified by the presence of primary growth oocytes (PG) and cortical aveolar oocytes (CA).

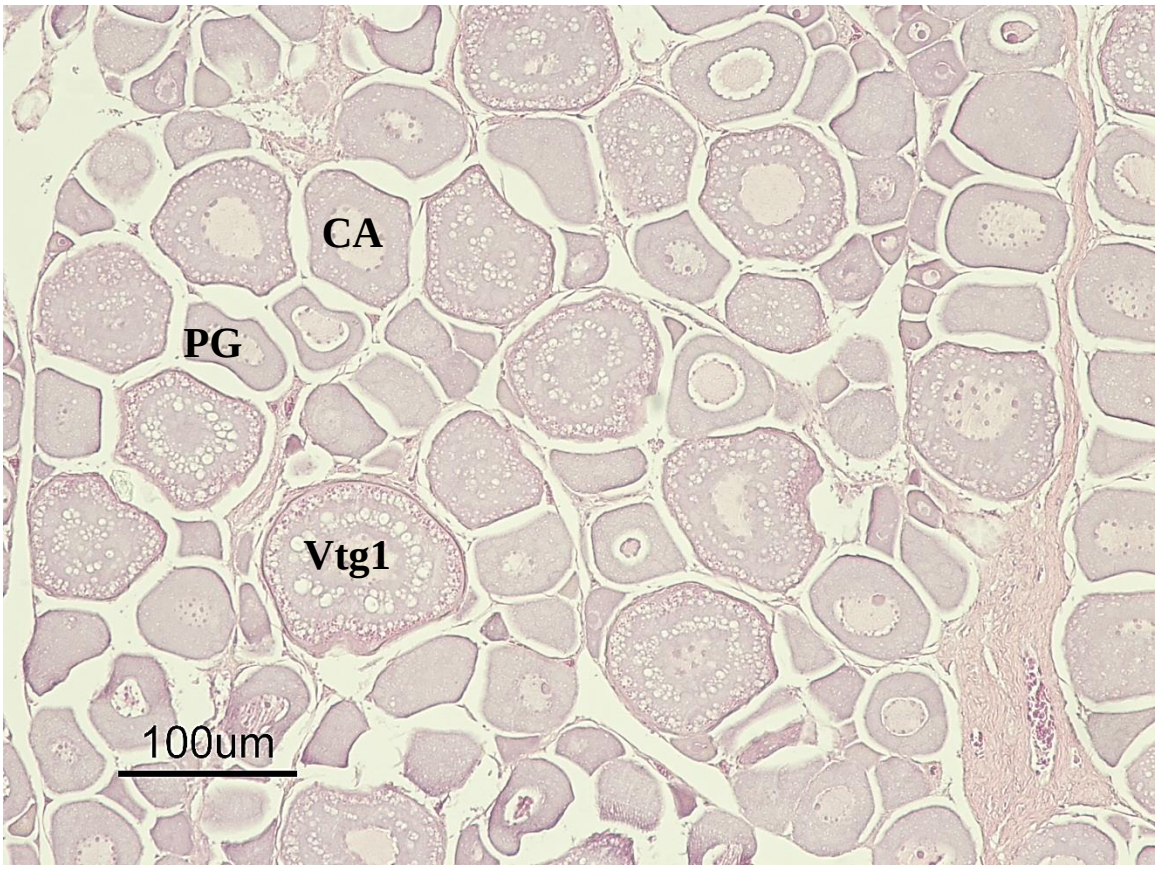

Figure 19. Developing female histology image

Histology sample imaged at 10x magnification from a developing female Southern Flounder caught in October 2014. Developing females were identified by the presence of primary vitellogenic oocytes (Vtg1) with some primary growth oocytes (PG) and cortical aveolar oocytes (CA).

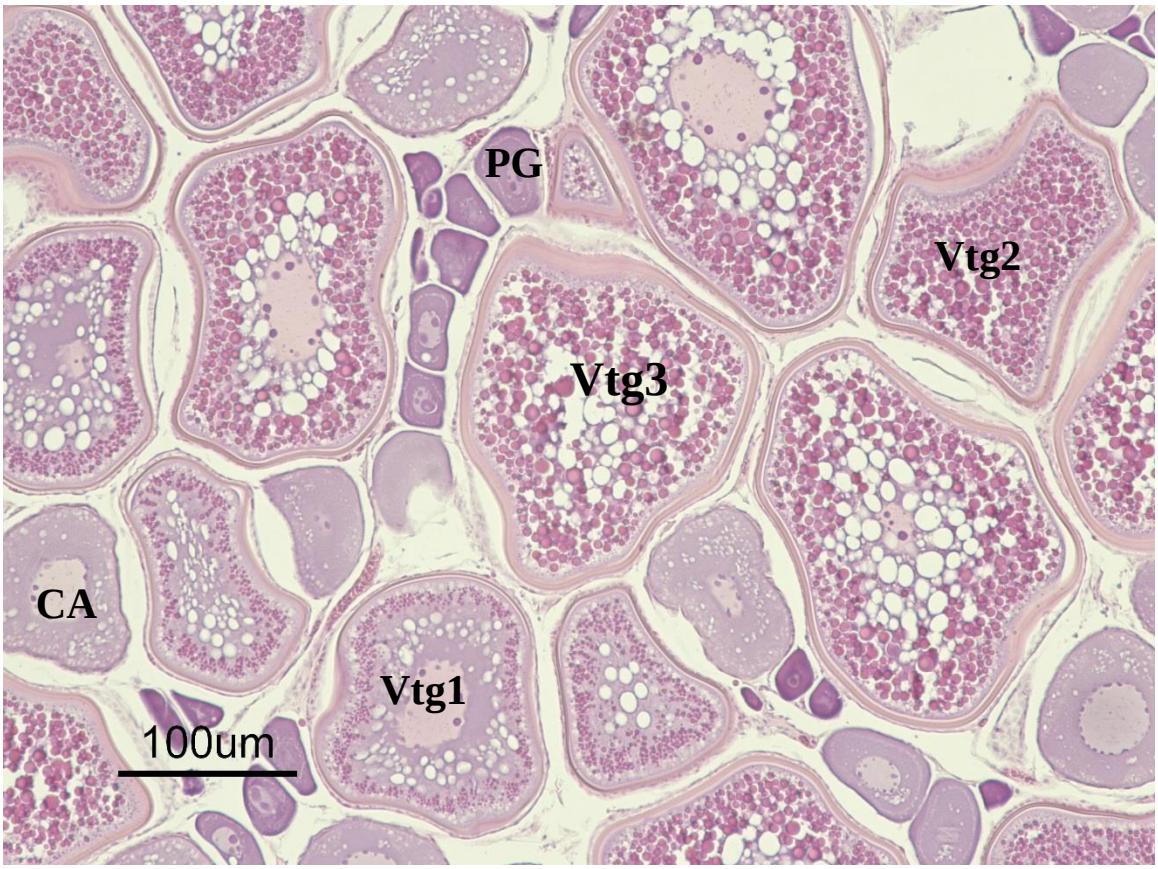

Figure 20. Spawning capable female histology image

Histology sample imaged at 10x magnification from a spawning capable female Southern Flounder caught in November 2015. Spawning capable females were identified by the predominance of tertiary vitellogenic ooctyes (Vtg3) with some primary (Vtg1) and secondary vitellogenic oocytes (Vtg2), primary growth oocytes (PG), and cortical aveolar oocytes (CA).

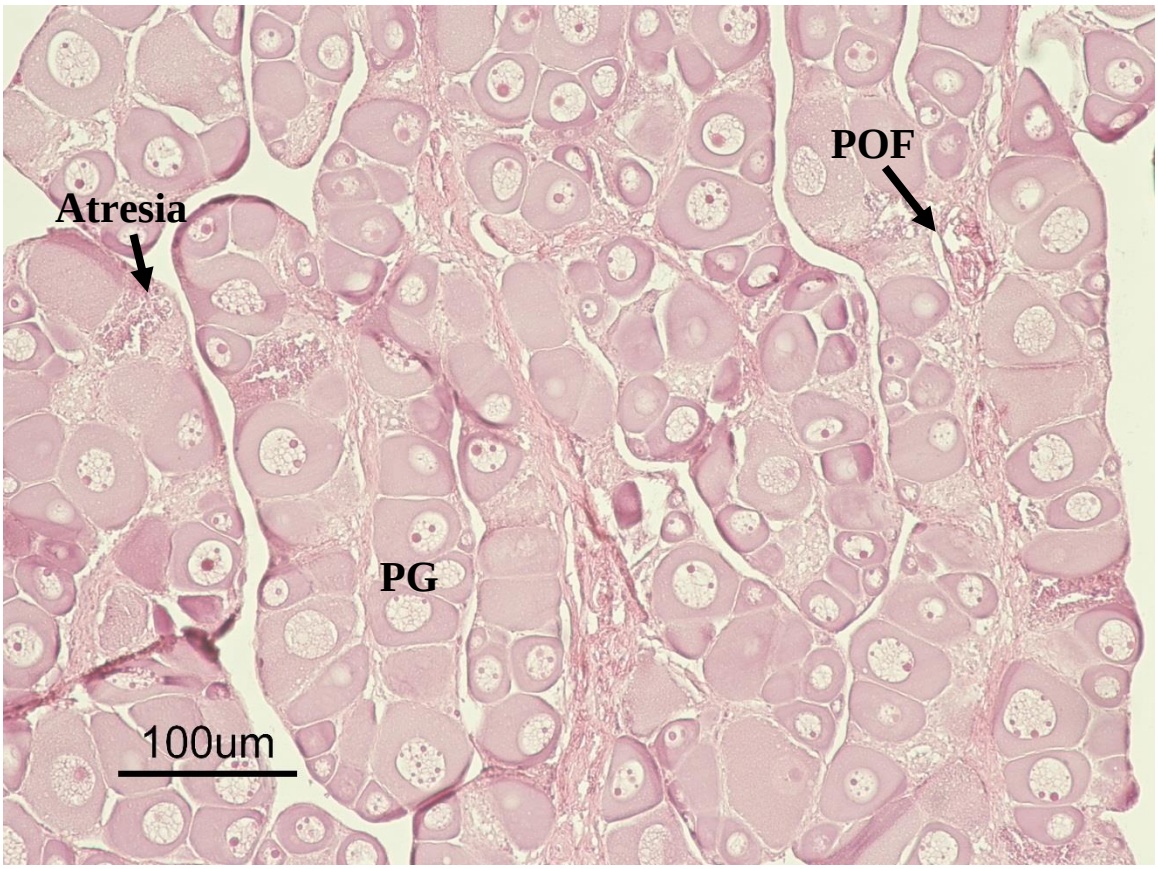

Figure 21. Regressing female histology image

Histology sample imaged at 10x magnification from a regressing female Southern Flounder caught in January 2016. Regressing females were identified by the presence of alpha atresia and post-ovulatory follicle complexes (POF) with primary growth oocytes (PG) predominant.

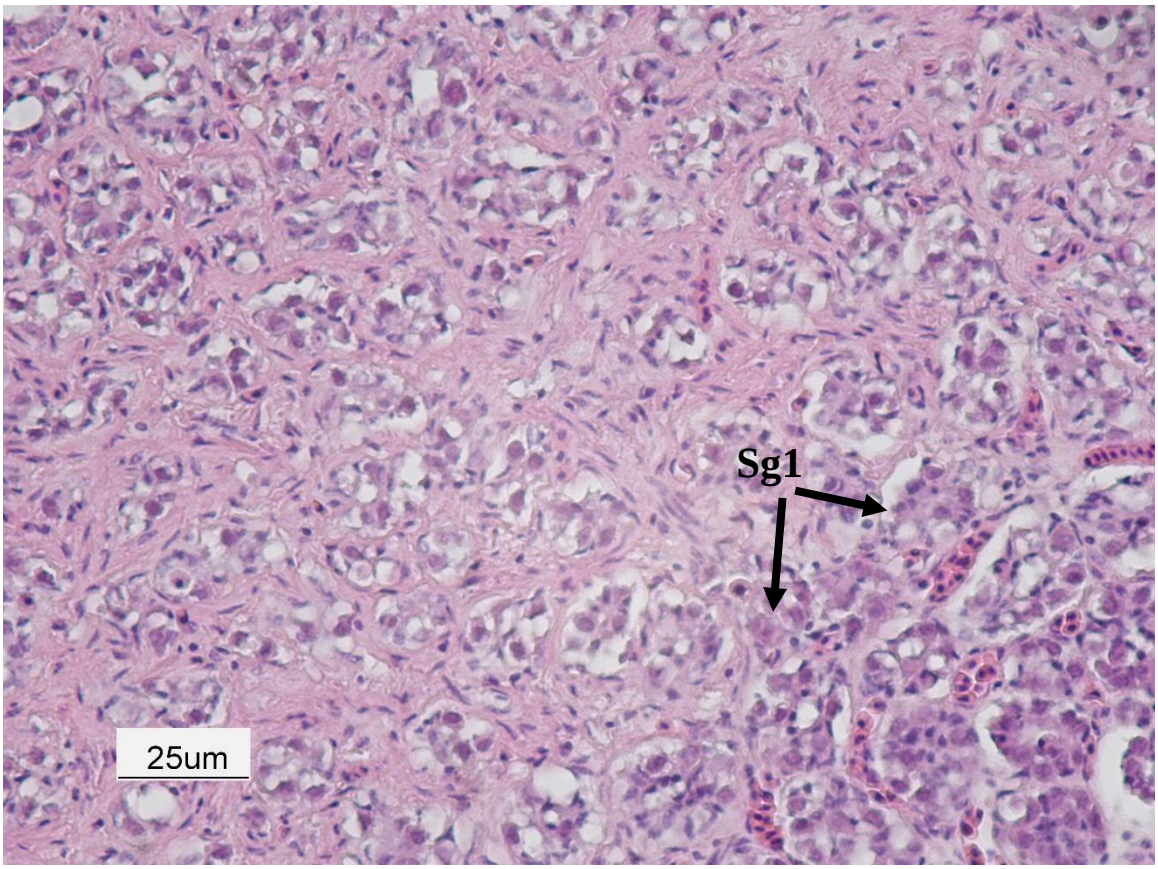

Figure 22. Immature male histology image

Histology sample imaged at 40x magnification from an immature male Southern Flounder (235 mm TL) caught in December 2014. Immature males were identified by the presence of primary spermatogonia (Sg1) and absence of lumens in the lobules.

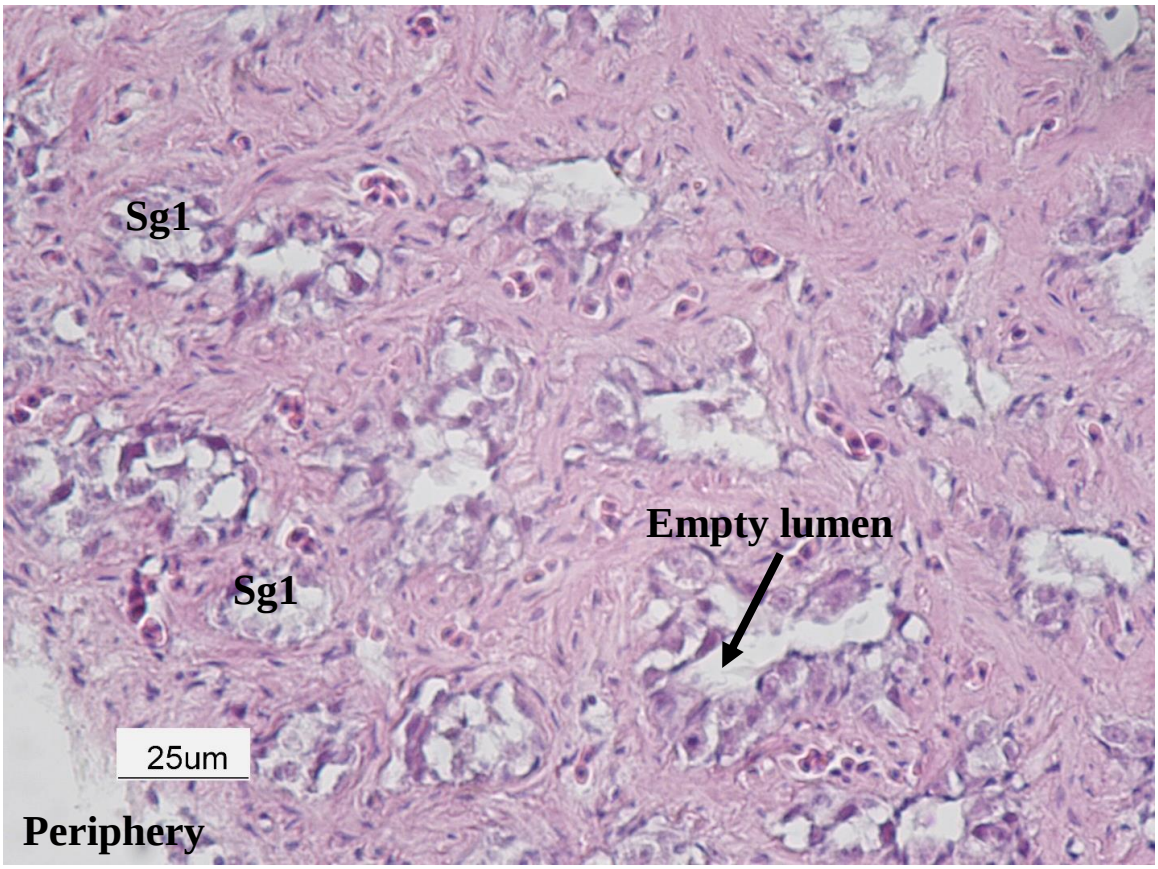

Figure 23. Regenerating male histology image

Histology sample imaged at 40x magnification from a regenerating male Southern Flounder caught in July 2015. Regenerating males were identified by the presence of empty lumens with proliferation of primary spermatogonia (Sg1) near the periphery.

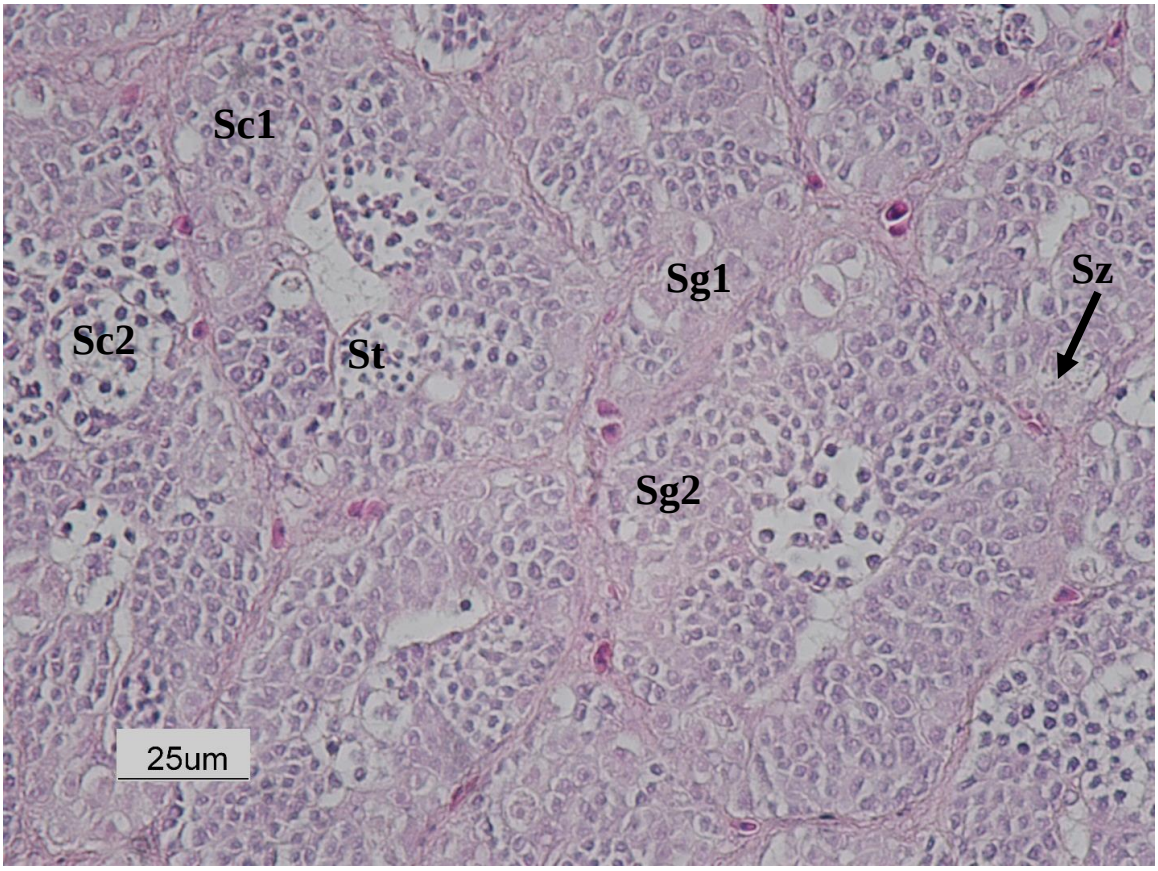

Figure 24. Developing male histology image

Histology sample imaged at 40x magnification from a developing male Southern Flounder caught in September 2014. Developing males were identified by the presence of all stages of spermatogenesis, including primary (Sg1) and secondary spermatogonia (Sg2), primary (Sc1) and secondary spermatocytes (Sc2), spermatids (St), and the absence of spermatozoa (Sz) in the lumens of lobules.

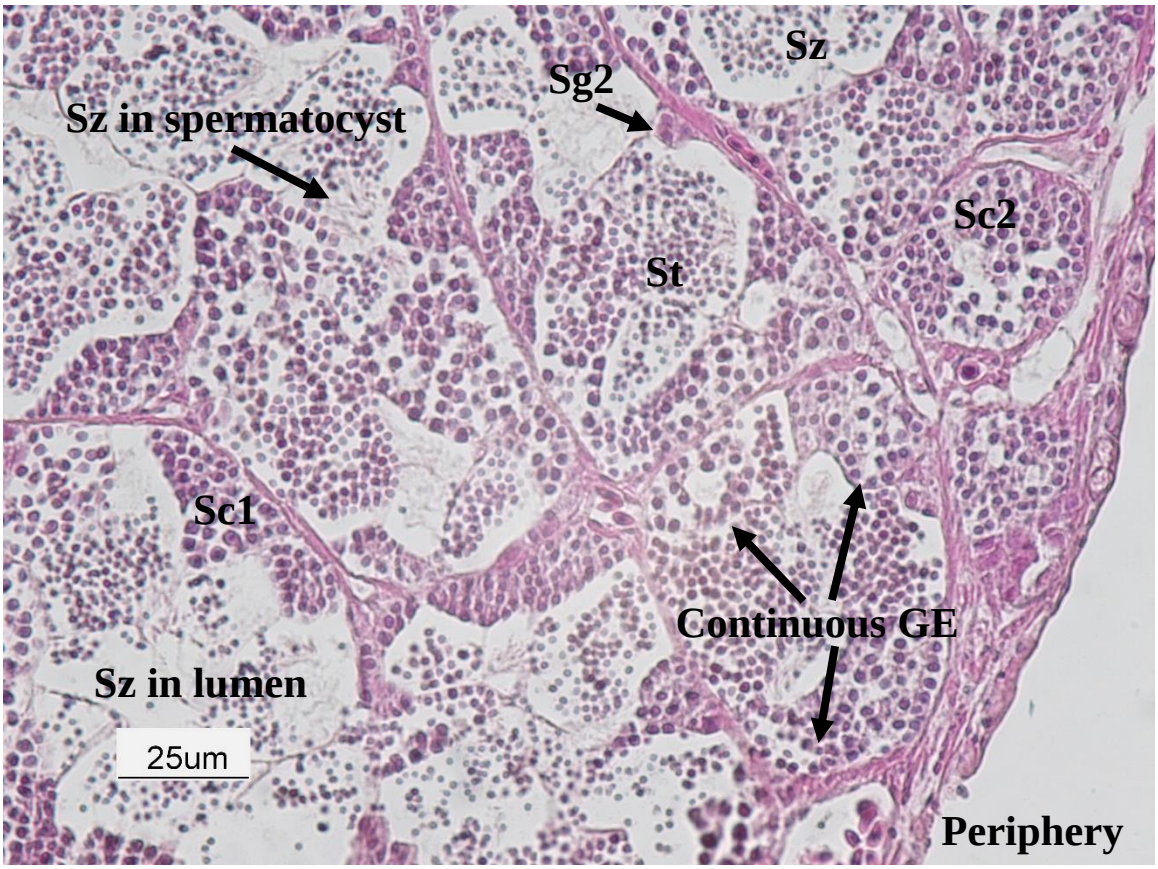

Figure 25. Early spawning capable male histology image

Histology sample imaged at 40x magnification from an early germinal epithelium (EGE) subphase spawning capable male Southern Flounder caught in December 2014. EGE spawning capable males were identified by the presence of spermatozoa (Sz) both in spermatocysts and in the lumen, and by a continuous GE throughout the testes. Other stages of spermatogenesis were also observed, including secondary spermatogonia (Sg2), primary (Sc1) and secondary spermatocytes (Sc2), and spermatids (St).

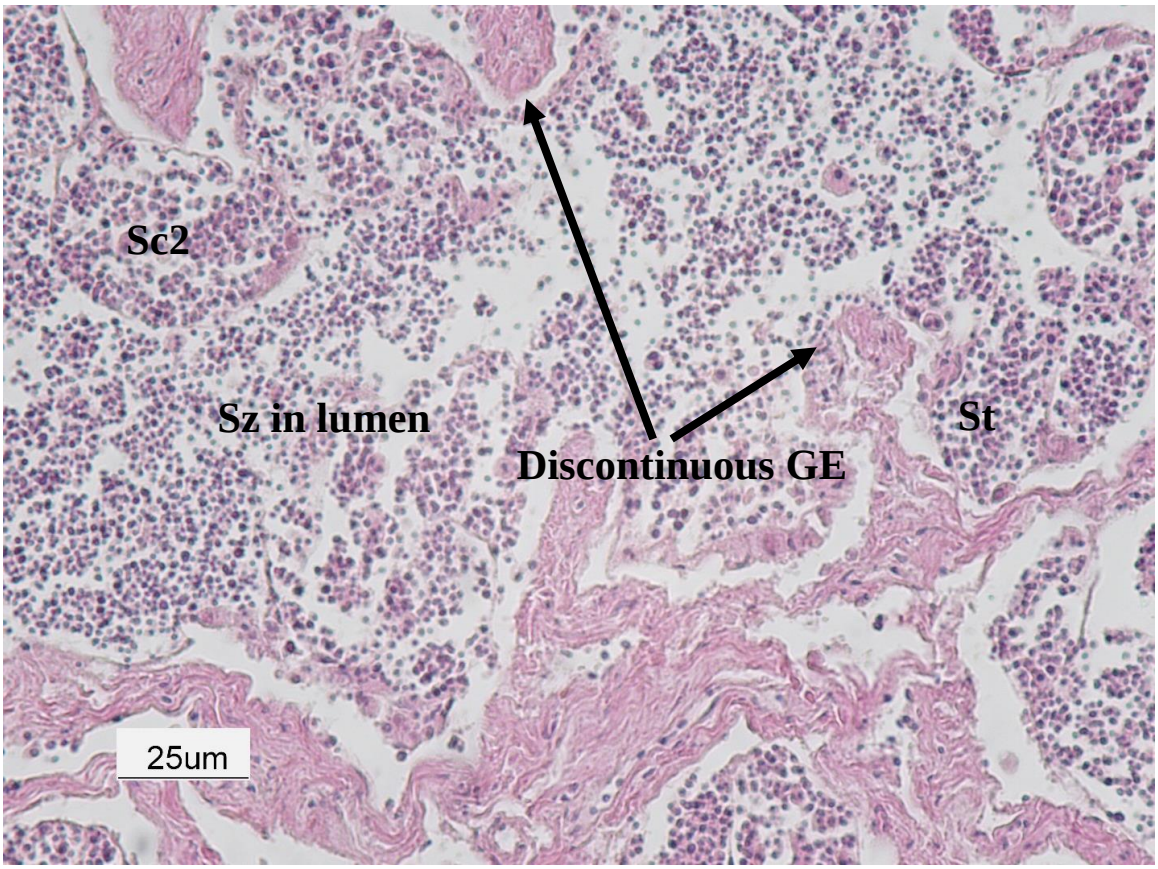

Figure 26. Late spawning capable male histology image

Histology sample imaged at 40x magnification from a late germinal epithelium (LGE) subphase spawning capable male Southern Flounder caught in December 2014. LGE spawning capable males were identified by the presence of spermatozoa (Sz) in the lumen and a discontinuous GE throughout the testes. Other late stages of spermatogenesis were also observed, including secondary spermatocytes (Sc2) and spermatids (St).

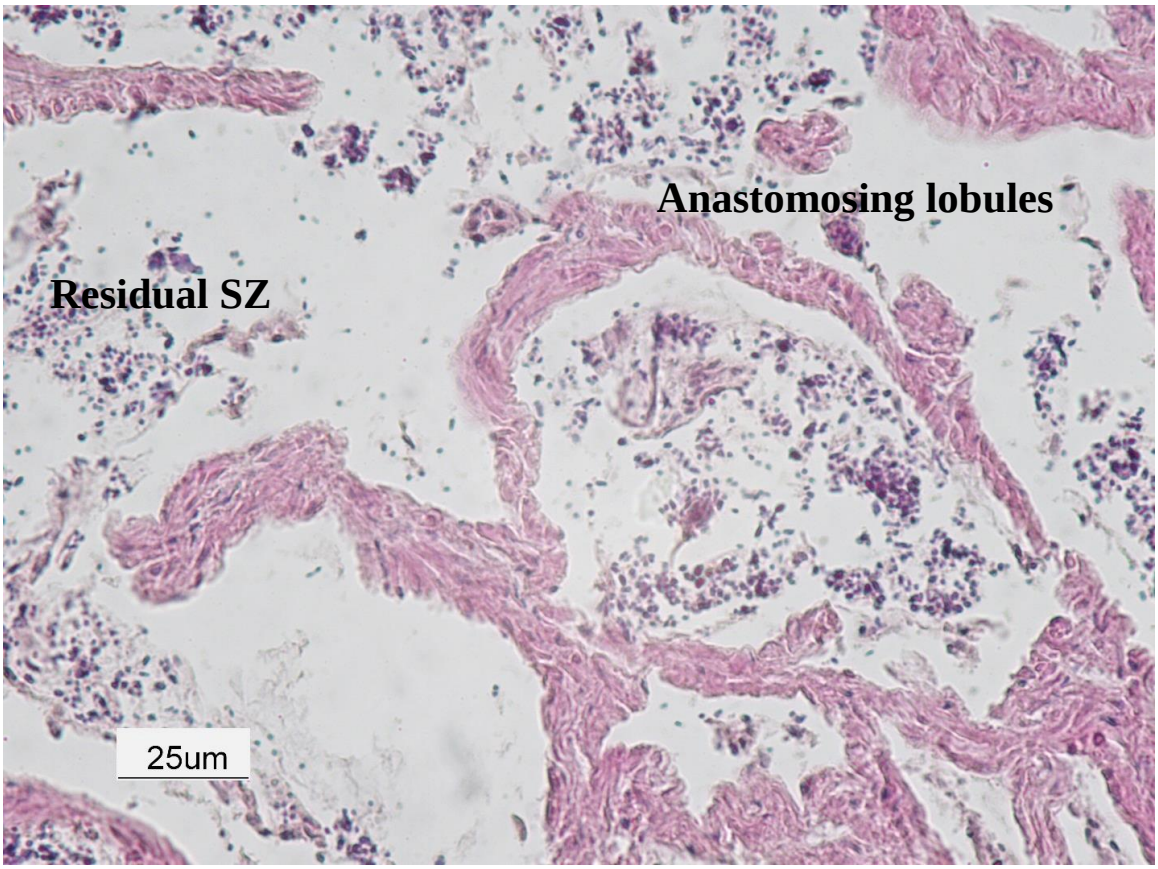

Figure 27. Regressing male histology image

Histology sample imaged at 40x magnification from a regressing male Southern Flounder caught in February 2015. Regressing males were identified by the presence of residual spermatozoa (Sz) and anastomosing lobules.

CHAPTER IV – CONCLUSION

Research on Southern Flounder life history conducted in the last two decades has focused on the Louisiana (Fischer and Thompson 2001, Fischer and Thompson 2004) and Texas stocks (Stunz et al. 2000, Glass et al. 2008, Nims and Walther 2014). In the most recent Fisheries Management Plan published by the Gulf States Marine Fisheries Commission, data from Louisiana and Texas stocks were used to represent the entire Southern Flounder fishery in the Gulf of Mexico (GOM) (GSMFC 2015). The scarcity of species-specific data in the north-central GOM has historically prevented a Gulf-wide stock assessment for the Flounder fishery. By describing growth and reproduction of Southern Flounder in the north-central GOM, this study contributes valuable information to inform future stock assessments and improve state-level management of the Mississippi stock. In conclusion, I will summarize the information presented in previous chapters and discuss management considerations for the Flounder fishery.

In Chapter II, I report Southern Flounder age and growth dynamics in the north-central GOM and length-at-age parameters specific to the Mississippi stock. Results from this research include validation of annuli deposition frequency and an examination of factors that influence deposition rates in age-one Southern Flounder otoliths. Following age estimation, I described the length-at-age and weight-at-length relationships for female Southern Flounder and compared mean parameter estimates to those reported in previous studies. There were no significant differences observed between length-at-age parameter estimates reported in the GOM, although there was spatial variability observed. Finally, I evaluated temporal variation in relative condition and found that

intra-annual relative condition did not greatly vary. The age and growth dynamics of Southern Flounder in Mississippi were previously undescribed. Given that Southern Flounder is a state-managed fishery and growth is variable within the GOM (Midway et al. 2015), local estimates of growth parameters are critical for evaluating population productivity and accomplishing management objectives.

In Chapter III, I describe the reproductive biology of Southern Flounder in the north-central GOM. Specifically, this study includes an estimation of female-specific length- and age-at-50% maturity parameters and a report of first maturity for females and males. Although length-at-age parameters did not greatly differ among GOM states, estimated length-at-50% maturity was significantly different from previously reported parameters in Louisiana. After describing maturation, I estimated the duration of the Southern Flounder spawning season using GSI data and histological phase classification. Finally, a histological description of gonadal development for both sexes and a classification of Southern Flounder spawning frequency were reported. The absence of actively spawning individuals captured during the sampling period is a major limitation to understanding the reproductive potential of this species. Nonetheless, this study represents the most recent comprehensive review of Southern Flounder reproduction in both the GOM and the Atlantic.

Southern Flounder exhibit a seasonal migration for spawning, and the spatial dynamics of this estuarine-dependent species have implications for management (Secor 2005). Although I was able to estimate spawning seasonality in this research, I did not collect evidence of where spawning occurred at offshore locations. Given the uncertainty

surrounding offshore migration in terms of spawning habitats used, the degree of mixing between state-managed stocks is undefined. Recently, there has been interest in understanding the stock structure of Southern Flounder. Past research has focused on using genetics (Blandon et al. 2001, Anderson et al. 2012), otolith morphometrics (Midway et al. 2014), tagging methods (Furey et al. 2013, Craig et al. 2015), and models of growth variability (Midway et al. 2015) to better describe Southern Flounder stock structure. Genetic studies reported that there is homogeneity in the Southern Flounder population with little structuring among or within states in the GOM (Anderson et al. 2012), and similar observations were reported based on otolith morphometric variation (Midway et al. 2014). In a tag-recapture study examining habitat use and movement patterns of Southern Flounder in the Atlantic, limited movement was reported during the spring and summer while fish resided in estuaries and spring recaptures of estuarine residents were near initial release sites (Craig et al. 2015). These results indicate that mixing occurs offshore during spawning, but also that small-scale environmental factors may be drivers of variability in demographic traits, such as growth and reproduction, during estuarine residency. Monitoring of growth and reproduction is beneficial for fisheries management because shifts in these biological parameters may indicate a population-level response to changes in fishing pressure or the environment (Trippel 1995, Shin et al. 2004). Thus, there is a need for local estimates of life-history parameters that reflect the current state of the fishery to inform stock assessments and resulting management decisions.

My thesis research substantially contributes to the understanding of Southern Flounder life history in the north-central GOM. Because management regulations are variable within the GOM and there is offshore mixing among state-managed stocks, multiple state and federal administrations mange the Southern Flounder population (GSMFC 2015). By describing the age and growth dynamics and reproductive biology of a popular recreationally harvested species, this thesis provides a comprehensive examination of Southern Flounder life history with information specific to the Mississippi stock. I expect that the results reported will serve as a scientific basis for regulations by state management agencies in the GOM.

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APPENDIX A – Histology Processing

Table A1.

Histological processing sequence

Step	Solution	Duration
1	70% EtOH	hold
2	80% EtOH	1:00 hour
3	95% EtOH	0:40 hour
4	95% EtOH	0:40 hour
5	95% EtOH	0:40 hour
6	100% EtOH	1:00 hour
7	100% EtOH	1:00 hour
8	100% EtOH	1:00 hour
9	Xylene Substitute	1:00 hour
10	Xylene Substitute	1:00 hour
11	Xylene Substitute	1:00 hour
12	Paraplast Plus	0:40 hour
13	Paraplast Plus	0:40 hour
14	Paraplast Plus	0:40 hour

Processing was completed using a Shandon Histocentre 2 Processor.

Table A2.

Tissue staining process outline

Step	Solution	Duration
1	Xylene Substitute	3:00 min
2	Xylene Substitute	3:00 min
3	Xylene Substitute	3:00 min
4	100% EtOH	10 dips
5	100% EtOH	10 dips
6	95% EtOH	10 dips
7	95% EtOH	10 dips
8	80% EtOH	10 dips
9	80% EtOH	10 dips
10	50% EtOH	10 dips
11	Distilled Water	1:00 min
12	Hematoxylin 2	3:00 to 6:00 min
13	Water	rinse
14	Acid Water	2 dips
15	Water	rinse
16	Blueing Water	0:30 sec
17	Water	rinse
18	95% EtOH	10 dips
19	Eosin Y	1:00 to 1:30 min
20	Blot Blot Blot	N/A
21	95% EtOH	10 dips
22	95% EtOH	10 dips
23	95% EtOH	10 dips
24	100% EtOH	1:00 min
25	100% EtOH	1:00 min
26	100% EtOH	1:00 min
27	Xylene Substitute	1:00 min
28	Xylene Substitute	1:00 min
29	Xylene Substitute	1:00 min
30	Xylene Substitute	1:00 min

APPENDIX B – IACUC Approval Letter

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.4063 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**14091802**
PROJECT TITLE:	**"Project I.N.S.P.I.R.E"**
PROPOSED PROJECT DATES:	**9/2014-9/2017**
PROJECT TYPE:	**New**
PRINCIPAL INVESTIGATOR(S):	**Robert Leaf**
DEPARTMENT:	**Coastal Sciences**
FUNDING AGENCY/SPONSOR:	**Mississippi Tidelands Trust Fund Program**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2017**

Frank Moore, Ph.D.
IACUC Chair

09/30/2014
Date