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Seasonal physiological dynamics of maturing female southern flounder (*Paralichthys lethostigma*)

Casey A. Grieshaber[1], Stephen R. Midway[2,*], Frederick S. Scharf[1], Heather Koopman[1], J. Adam Luckenbach[3,4] and Mollie A. Middleton[5,]

[1]Department of Biology and Marine Biology, University of North Carolina Wilmington, Wilmington, NC 28403, USA
[2]Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, LA 70803, USA
[3]Environmental and Fisheries Sciences Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Seattle, WA 98112, USA
[4]Center for Reproductive Biology, Washington State University, Pullman, WA 99164, USA
[5]School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195-5020, USA

***Corresponding author:** Department of Oceanography and Coastal Sciences, Energy, Coast & Environment Building, Louisiana State University, Baton Rouge, LA 70803, USA. Tel: +1 225 578 6458. Email: smidway@lsu.edu

Physiological information is rarely used in descriptions of maturity for managed, wild fish species; however, the use of physiological data holds great promise to provide important detail on the complexities of oocyte development and maturity. Investigating southern flounder (*Paralichthys lethostigma*)—an overfished commercial and recreational fishery resource—we examined pre-spawn physiological changes in females to provide further detail of the maturation process. Given that adults of this species complete maturation and spawn in unknown offshore locations, information on pre-spawn physiological changes is particularly informative for both size- and age-based patterns of maturity. We evaluated seasonal and ontogenetic changes in hormone concentrations in blood plasma that are commonly associated with sexual maturation, in addition to quantifying and classifying lipid stored in liver tissue. We found a strong positive relationship between body weight and lipid content during all months, as well as evidence for mobilization of lipids among larger females in September and October, presumably for gonadal development. Throughout the sampling period, the lipid content of smaller individuals was dominated by structural lipids (as opposed to storage lipids). In contrast, larger individuals possessed greater amounts of storage lipids. This suggests that larger, putatively maturing individuals were accumulating storage lipids for later production of vitellogenin. Females sampled for blood sex steroids and ovarian histology showed different testosterone and estradiol concentrations between putatively maturing and immature fish, and temporal variation with peaks in October and November. Overall, emerging patterns of liver lipid content and composition and blood steroid concentrations describe a multi-month maturation process that is often managed one dimensionally over short time periods. Insights from this work will improve our understanding of the life history of southern flounder, with the potential for better understanding of the dynamics of offshore spawning migration and informing subsequent species management.

Key words: Lipid content, maturity, *Paralichthys lethostigma*, sex steroids

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Introduction

Reproduction is key to a species' persistence and involves many complex and specific processes that occur at an organismal level. In order to reproduce successfully, teleost fishes undergo several important physiological and physical changes. Two specific processes that occur in many teleosts include the accumulation of lipids—which are later mobilized for the production of vitellogenin (an egg yolk precursor protein) and the eventual development of oocytes—and changes in hormone concentrations, which drive sexual maturation (Tyler and Sumpter, 1996). In preparation for spawning, fishes begin to accumulate energy stores, mainly in muscle and liver tissues (Alonso-Fernández and Saborido-Rey, 2012). As spawning becomes more imminent, hormone changes occur that initiate the transfer of energy reserves into the ovaries of maturing female fishes and that also control spawning (Okuzawa 2002; Miller and Kendall, 2009). Furthermore, many fish undergo large seasonal changes in feeding and growth environments and may migrate long distances to spawn. These variable environments and reproductive investments can place considerable energetic demands on individuals, particularly when food is scarce (Jørgensen *et al.*, 2008). Ultimately, energetic demands are dependent on fish physiology, which means there may be spatial and temporal variability in fish condition (Lowerre-Barbieri *et al.*, 2011), and this variability may need to be considered by managers.

Past studies have shown that both hormone (Pavlidis *et al.*, 2000) and lipid (Malavasi *et al.*, 2004) concentrations are well correlated with the development of the gonads and subsequent reproduction in teleosts. For many teleost fishes, the liver has been shown to serve as an important storage location for lipids (Sheridan, 1988; Marshall *et al.*, 1999; Yaragina and Marshall, 2000). Wiegand (1996) further described that teleost livers contain varying concentrations of triacylglycerol (TAG), which is the form of lipid in which energy stores are maintained for future use. Fish also contain phospholipids (PL), which are normally used as structural lipids, most importantly in cellular membranes. Storage lipids are important in fish that are initiating maturation and are beginning to mobilize lipids for ovarian development, whereas structural lipids are important for maintaining basal metabolic function and may be seen as the most abundant lipid in fish that have not yet begun oocyte development for the current spawning season. During yolk formation, however, the importance of PL increases because a large fraction of the lipids in an egg occur in this form (Wiegand, 1996).

When fish initiate puberty or ovarian recrudescence, hormones (primarily estradiol) trigger and regulate production of liver vitellogenins (VTG), consisting of ~80% protein and ~20% lipid (mostly TAG), which are then transferred through the blood for uptake by the ovaries (Tocher, 2003; Lubzens *et al.*, 2010). Uptake of VTG is critical for continued oocyte maturation (Jørgensen and Fiksen, 2006) and is later necessary for embryonic and larval fish growth (Wiegand, 1996; Johnson, 2009). In fish that are preparing to mature, lipids are often stored throughout the year and later mobilized during gonadal maturation (Foltz and Norden, 1977), thus lipids are essential for oocyte maturation and successful reproduction (Jørgensen and Fiksen, 2006). Lipid stores have been linked to total egg production and recruitment success (Marshall *et al.*, 1999; Jørgensen and Fiksen, 2006), and thus are proving to be an important physiological tool in understanding population-level reproductive health.

Concurrent with changes in somatic lipid stores, female fish undergo fluctuations in reproductive hormones. Hormonal changes in fish regulate sexual maturation and eventually result in spawning-capable individuals. Similar to most vertebrates, female teleosts produce gonadotrophin-releasing hormone that is released from the hypothalamus and triggers the production of the gonadotrophins, luteinizing hormone and follicle-stimulating hormone, by the pituitary gland (Idler and Ng, 1983; Zohar *et al.*, 2010). Gonadotrophins travel through the blood and bind to their receptors in the ovary. This triggers increased production of testosterone (T), which is converted to 17β-estradiol (E_2) to promote maturation (Idler and Ng, 1983). Testosterone and E_2 concentrations are often highest during the vitellogenic stage of ovarian development, which is also the stage when many species are classified as sexually maturing or developing (Kjesbu *et al.*, 2003; Brown-Peterson *et al.*, 2011). The use of blood hormones to inform the timing of reproductive events and their implications for harvest and management has historically been limited in fisheries science.

The southern flounder (*Paralichthys lethostigma*) is a flatfish that inhabits estuarine and coastal waters of the western Atlantic Ocean and Gulf of Mexico (Gilbert, 1986). Beginning in the autumn, maturing fish migrate offshore to complete maturation, and spawning is thought to take place annually between December and March (Wenner *et al.*, 1990). The vast majority of female southern flounder reach maturity by age 3 years (Midway and Scharf, 2012), and the fishery harvests mainly 1- and 2-year-old fish, which represents the immature fish that populate natal estuaries. Given the high rates of exploitation in recent years (Smith *et al.*, 2009) and the potential for environmentally driven interannual variation in the timing of maturity and/or spawning participation (such as changes in temperature and earlier maturation; Atkinson, 1994), an improved understanding of the seasonal physiology of southern flounder might improve the effectiveness of management strategies. For example, the ability to estimate the annual proportion of maturing females before the offshore autumn emigration could enable adjustments to exploitation rates that would ensure sufficient escapement. The North Carolina Division of Marine Fisheries (NCDMF, 2009) reports that southern flounder are the most economically important estuarine finfish species in North Carolina. However, the North Carolina stock is

currently in a 'depleted' state. Owing to their temporal accessibility and economic importance, and successful recent studies on similar flatfishes (e.g. Barnett and Pankhurst, 1999; Merson *et al.*, 2000), southern flounder represent a good model species in which to explore temporal patterns of sex steroids and changes in lipid accumulation and composition in relationship to sexual maturation, with the ultimate goal of improved understanding of the maturation process for species conservation and management.

The objectives of this study were as follows: (i) to describe concentrations of blood/plasma sex steroids during early maturation in conjunction with other indicators of maturation [e.g. gonadal histology, gonadosomatic index (GSI) and hepatosomatic index]; (ii) to describe seasonal and size-based patterns in lipid accumulation in the liver; and (iii) to consider how both of these changes impact our understanding of the timing of reproduction in southern flounder.

Materials and methods

Quantifying blood hormone concentrations and associated reproductive state

Fish capture and blood sampling procedures

The first set of southern flounder was collected using gill nets in Masonboro Sound, NC, USA during September–December 2011 (note that the fish analysed for blood chemistry were separate from the fish sampled for lipid analysis). Short gillnet soak times (<8 h) and cool autumn water temperatures resulted in high survival rates (>95%) at the time of gear retrieval. Fish were maintained alive in a circulating aquarium for a short recovery period (1–2 days) and then sedated with clove oil (80 mg/l; Borski and Hodson, 2003). Total length [TL (in millimetres)] and mass (in grams) were recorded, and a sample of blood (approximately 3–5 ml) was collected from the caudal vein using a 21-gauge sterilized needle and syringe (Strange, 1996). Blood samples were then transferred into heparinized BD Vacutainer™ tubes (BD Vacutainer Systems, Franklin Lakes, NJ, USA) and placed on ice for no longer than 6 h, but frequently <1 h, to help minimize storage effects on blood hormones or proteins (Clark *et al.*, 2011). Plasma was separated by placing 1 ml of blood into two vials and centrifuging the vials at 10 000 rpm for 7 min. Plasma was then partitioned into three 0.3 ml aliquots and placed in an ultracold freezer (−80°C) until all samples were analysed.

Plasma steroid analysis

Steroids were extracted from southern flounder plasma by double-ether extraction in a similar manner to Merson *et al.*, (2000). Briefly, 1.5 ml of ether was added to 150–400 µl of plasma from each fish and vortexed for 15 s. For the second extraction, 1 ml of ether was added. Supernatants from both extractions were pooled and dried down in a 37°C water bath with N_2 gas. The extract was reconstituted in an equivalent volume of assay buffer for the T (150 µl) and E_2 (400 µl) assays. Plasma T concentrations were determined by enzyme immunoassay (EIA) using an antibody validated by Rodríguez *et al.* (2000) and provided by Dr Sylvia Zanuy (Instituto de Acuicultura de Torre la Sal, Castellón, Spain). Testosterone conjugated with acetylcholine esterase was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), and the EIA was performed as previously described (Rodríguez *et al.*, 2000). Plasma E_2 concentrations were determined by radioimmunoassay (RIA) as previously described (Sower and Schreck, 1982).

Four plasma samples from southern flounder with the highest GSI [(gonad mass/body mass) × 100] were pooled, and a dilution curve was generated. The southern flounder plasma dilution series showed parallelism to standard curves of both the T and E_2 assays, indicating that the assays were valid for measuring these steroids in southern flounder plasma (see Supplementary material, Fig. S1). All southern flounder plasma samples from the study fell within the linear range of the flounder plasma curves. For T, the inter-assay variation was 13.7% and intra-assay variation 4.1%. For E_2, the inter-assay variation was 13.8% and intra-assay variation 2.0%.

Gonad histology

After blood collection, fish were euthanized (in MS-222) and gonad and liver weight measured. Whole gonads were placed in 10% neutral buffered formalin for a minimum of 4 weeks prior to histological processing of a central section of the gonad. Histological processing was performed using standard methods, involving ethanol and xylene rinses followed by wax paraffin embedding. After hardening, 5-µm-thick sections were cut from the embedded sample block, mounted on glass slides and stained with Gill's haematoxylin #2 and eosin-Y.

Histological samples were staged using two separate criteria, each with different designations of maturity. For both criteria, the stages included primary growth (containing no yolk or cortical alveoli), cortical alveolus (presence of cortical alveoli vesicles/yolk proteins) and vitellogenesis (increase in yolk granules and oocyte size; Murua, 2003). The first set of criteria staged oocytes based on the most advanced oocyte stage present, with the presence of cortical alveolus or vitellogenic stage oocytes indicating maturity within the present season (hereafter, conventional criteria; Murua, 2003; Brown-Peterson *et al.*, 2011). The second set of criteria for staging the ovaries was also based on the most advanced oocyte stage present; however, this staging method (hereafter, modified criteria) broke down the cortical alveolus stage into early and late cortical alveolus (Fig. 1). Exact thresholds for oocyte development and the determination of whether or not an individual will mature are under continued investigation, and thus our evaluation of early and late cortical alveoli represents a best guess. Although the oocyte stage associated with commitment to an upcoming spawning

season has not yet been determined for southern flounder, in captive studies of Atlantic cod, some individuals with low or reduced levels of 17β-estradiol tended to halt development during the early cortical alveoli stage and, subsequently, to reabsorb those oocytes via follicular atresia (Kjesbu *et al.*, 2011; Rideout and Tomkiewicz, 2011). As a result of this, we have broken the cortical alveoli stage down into early and late stage cortical alveoli. Although a number of changes occur between these two phases of oocyte growth, we focused on the presence of a well-defined zona radiata. The zona radiata (also known as the zona pellucida, chorion or vitelline envelope) has been shown to develop during the cortical alveolus stage (Patiño and Sullivan, 2002), and thus allowed for early and late stage designations based on the presence or absence of this feature. Using this modified staging scheme, early cortical alveolus stage oocytes were considered immature, whereas late cortical alveolus stage oocytes were considered maturing. Using both criteria, specimens were assigned oocyte stages by two independent readers. The assignment of oocyte stages ultimately allowed for fish to be categorized as either immature or maturing under each staging scenario.

Both left and right otoliths were extracted, and annuli counts were made separately by two readers using whole otoliths to estimate fish age, using methods similar to those of Wenner *et al.* (1990).

Lipid content and composition

A second set of southern flounder was collected by fishery-independent gillnet sampling conducted by NCDMF staff between 3 July and 12 December 2012 and used to analyse seasonal and size-dependent patterns in lipid storage and composition. Livers were removed at the time of capture and stored at approximately −20°C. A subset of livers ($n = 125$, all from females) was randomly selected for analysis using fish size and collection month as strata. Lipid extraction followed the 2:1 chloroform:methanol method described by Folch *et al.* (1957) using samples of ~1 g of liver tissue to determine lipid content, expressed as the percentage wet weight. Lipid was then transferred into storage vials, into which 100 mg/ml of hexane was added. Vials were then stored at −20°C for subsequent lipid classification.

To determine the types of lipids being stored in the livers of southern flounder, lipid classes [triacylglycerol (TAG), wax esters (WE), free fatty acids, cholesterol and phospholipids (PL)] were identified and quantified using automated thin-layer chromatography with flame ionization detection. Samples were spotted into chromarods, developed in a solvent system of 94/6/1 hexane/ethyl acetate/formic acid, and analysed with a Mark VI Iatroscan (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). Lipid class peaks (as weight percentage of total lipids) were integrated and quantified using Peak Simple 329 Iatroscan software (SRI Instruments, Torrance, CA, USA). Identification of peaks was confirmed using known standards (NuChek Prep, Elysian, MN, USA) of WE, TAG, free alcohols, PL and cholesterol (Koopman, 2007). Each sample was run in duplicate, and results were averaged. Triacylglycerol and PL were the focus of our statistical analyses because of their importance as storage and structural lipids, respectively (Wiegand, 1996).

Statistical analyses

To test for differences in steroid concentrations between putatively maturing and immature fish under both staging criteria, we used Student's *t*-test for groups with unequal variance. We suspected that the variances for the groups would differ, although there is no effect on parameter estimation if the variances do not differ. We used Bayesian estimation to arrive at parameter estimates, in order to take advantage of deriving quantities for the difference in means. Bayesian methods allow us not only to calculate parameter estimates, but also to provide estimates of uncertainty (credible intervals) for stronger inference. For our analysis, all parameters were given diffuse Normal priors. We ran three concurrent Markov chains, beginning each with a randomly generated value. The first 5000 iterations of each chain were discarded as burn-in, and the remaining 5000 were assessed for convergence with the Brooks-Gelman–Rubin statistic, $\hat{R}$. Conventionally, values <1.1 indicate convergence; we recorded no values >1.01. Analyses were run using JAGS (Version 4.1.0) in the rjags package (Plummer, 2015), run from within R.

To determine how other gross features related to maturity, correlation analysis (Spearman's ρ) was conducted between the GSI, HSI [(liver mass/body mass) × 100] and T and E_2 concentrations, respectively. Although there currently exist no constraints on sacrificing southern flounder for maturity investigation, the use of hormones as non-lethal maturity predictors has been evaluated in other species (e.g. Heppell and Sullivan, 1999). To this end, we explored the use of linear discriminant functions to determine the improvement in maturity prediction based on non-lethal models. Jackknife reclassification success was used to evaluate model performance. Owing to unequal sample sizes and problems related to chance classification (White and Ruttenberg, 2007), we set discriminant function group sizes equal to the smallest group. We ran 10 000 randomizations to account for individuals in the largest group; a high number of randomizations would include all individuals in large groups and, ultimately, provide a better description of that group. With this approach, we were able to explore a large suite of models for potential use in predicting maturity. In all models, maturity designation was based on the modified criteria, in order to provide greater separation between groups.

Given that maturity data were not recorded by NCDMF staff for the fish sampled for lipid analysis, we used fish

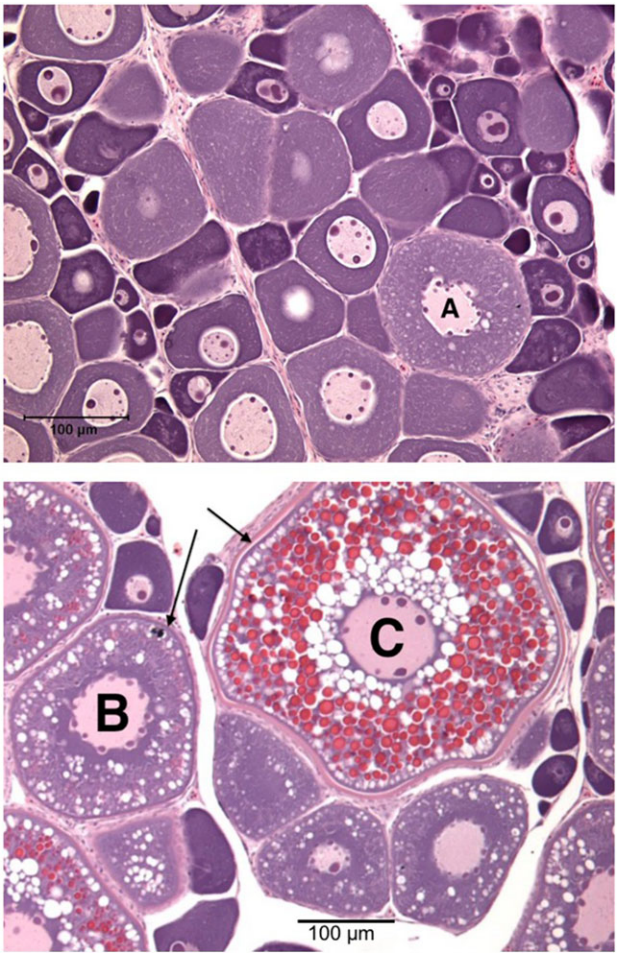

Figure 1: Representative histological samples illustrating early and late cortical alveolus stage oocytes of southern flounder. In the top panel, 'A' identifies an early cortical alveolus stage oocyte that would indicate development (and thus putative initiation of maturity) under the conventional staging criteria. In the bottom panel, 'B' shows a late stage cortical alveolus oocyte, in which an arrow indicates the still developing but visible zona radiata. Although oocyte 'C' is vitellogenic and advanced beyond the cortical alveolus stage, the arrow associated with 'C' illustrates a more developed zona radiata (along with three early cortical alveolus stage oocytes below 'C').

length and weight to predict maturity. The model we used was as follows:

$$P(\text{maturity}) = \frac{1}{1 + e^{-[4.06 + (-0.032 \times \text{TL}) + (0.010 \times W)]}}$$

where TL is the total length of the fish (in millimetres) and W is the weight of the fish (in grams). Fish were then classified as immature if the estimated probability of maturity was <0.50 and classified as putatively maturing if the estimated probability of maturity was >0.50. This predictive approach follows a recent study (Midway *et al.*, 2013), in which a larger number of models was explored (using >400 histologically validated fish) to predict southern flounder maturity in the absence of explicit maturity data. We did not assign putative maturity status to the summer fish because the predictive models (Midway *et al.*, 2013) were built and tested using characteristics of southern flounder captured only during the autumn reproductive season. The model we used in this study was successful in predicting maturity for 78% of fish based on leave-one-out reclassification and was the best-performing model which used the limited inputs (TL and W) available during the present study.

Both liver lipid content (percentage weight) and lipid class composition (weight percentage of total lipids) were examined for their dependence on time of year (date of capture), body size (weight [in grams]) and predicted maturity stage. Fish captured in July and August were considered to represent the summer period, whereas individuals caught during October and November were assigned to the autumn category. Both liver lipid content and the levels of PL and TAG were examined for changes between summer and autumn periods. The relationship between the levels of PL and TAG and total liver lipid content was also examined to determine how lipid composition varied with changes in total lipid content. We tested for an apparent threshold (body mass ~1 kg) by comparing a linear model and a segmented regression (from the segmented package in R, which uses maximum likelihood estimation).

Results

Blood hormone concentrations and reproductive state

During autumn 2011, 103 female southern flounder were collected to examine blood hormone concentrations and associated reproductive state. Fish ranged in length from 310 to 512 mm TL (mean TL = 382 mm, SD = 31 mm) and in mass from 348 to 1940 g (mean = 681.7 g, SD = 207.62 g). The vast majority of fish (n = 90) were determined to be 1 year old. Throughout the study period, concentrations of plasma E_2 ranged from 0.011 to 0.225 ng/ml and T ranged from 0.030 to 0.202 ng/ml. Although mean steroid concentrations varied through time, they peaked in early November, followed by a slow decline through December (Fig. 2).

Conventional histological staging identified 67 immature and 36 maturing fish, whereas the modified staging criteria that included both early and late cortical alveolus stages identified 79 immature and only 24 maturing fish. For both E_2 and T, the conventional histological staging criteria provided separation between means, although the separation was greater for E_2, where 95% credible intervals (95% CI) did not overlap (Table 1 and Fig. 3). The modified staging criteria provided even greater separation of means for both hormones. For E_2, the average difference between means went from 0.036 (95% CI: 0.015, 0.057) to 0.061 (95% CI: 0.036, 0.086). The group means for T also increased in their

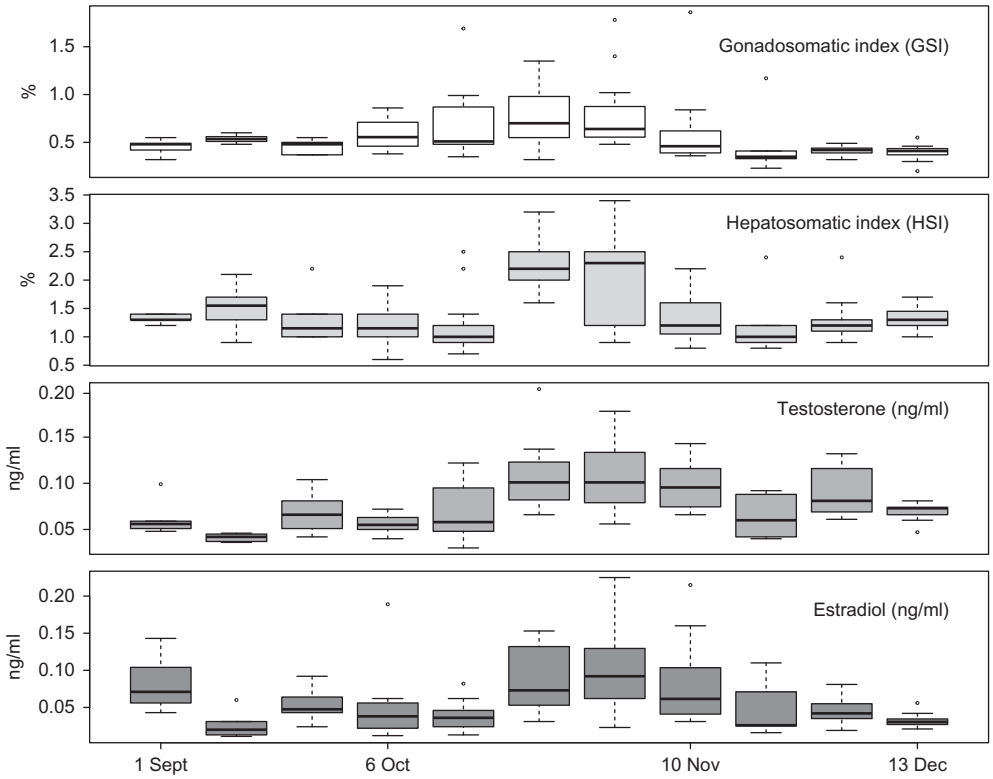

Figure 2: Gonadosomatic index (as a percentage), hepatosomatic index (as a percentage), testosterone (in nanograms per millilitre) and estradiol (in nanograms per millilitre) measured in female southern flounder collected weekly throughout the autumn of 2011. Boxes represent the interquartile range, and the bar within each box represents the mean. Whiskers extend to 1.5 times the interquartile range, and individual dots represent extreme values.

Table 1: Parameter estimates (with 95% credible intervals in parentheses) from Student's *t*-test using Bayesian estimation

Hormone	Parameter	Conventional staging (ng/ml)	Modified staging (ng/ml)
Estradiol	μ_1	0.048 (0.040, 0.056)	0.046 (0.040, 0.053)
Estradiol	μ_2	0.084 (0.065, 0.104)	0.107 (0.083, 0.132)
Estradiol	$\mu_2-\mu_1$	0.036 (0.015, 0.057)	0.061 (0.036, 0.086)
Testosterone	μ_1	0.074 (0.068, 0.080)	0.074 (0.068, 0.080)
Testosterone	μ_2	0.092 (0.078, 0.105)	0.099 (0.080, 0.118)
Testosterone	$\mu_2-\mu_1$	0.017 (0.003, 0.032)	0.025 (0.005, 0.044)

Symbols: μ_1, immature; μ_2, putatively mature; and $\mu_2-\mu_1$ represents the the difference in means. Although no *P*-values are produced, overlapping of the difference in means with zero could be interpreted as non-significant. Here, all distributions of differences in mean do not overlap zero, and also increased under the modified staging criteria.

difference under the modified staging criteria, although less so than E_2 (Fig. 3).

The GSI ranged from 0.20 to 1.86%, with a mean of 0.47% for immature fish and 1.00% for maturing fish, based on conventional maturity staging. Mean GSI values of all fish increased through late October and returned to low levels by late November (Fig. 2), although no maturing fish (using the modified criteria) were encountered in September and only five were observed in December. The HSI ranged from 0.40 to 3.40%, with a mean of 1.3% for immature fish and 2.3% for maturing fish. The seasonal pattern of mean HSI was generally similar to the pattern for GSI, but the seasonal peak in HSI was more punctuated during late October/early November (Fig. 2). Correlations between blood hormone concentrations and liver and gonadal indices were positive, and strongest between GSI and HSI, HSI and E_2, and T and E_2 (Table 2).

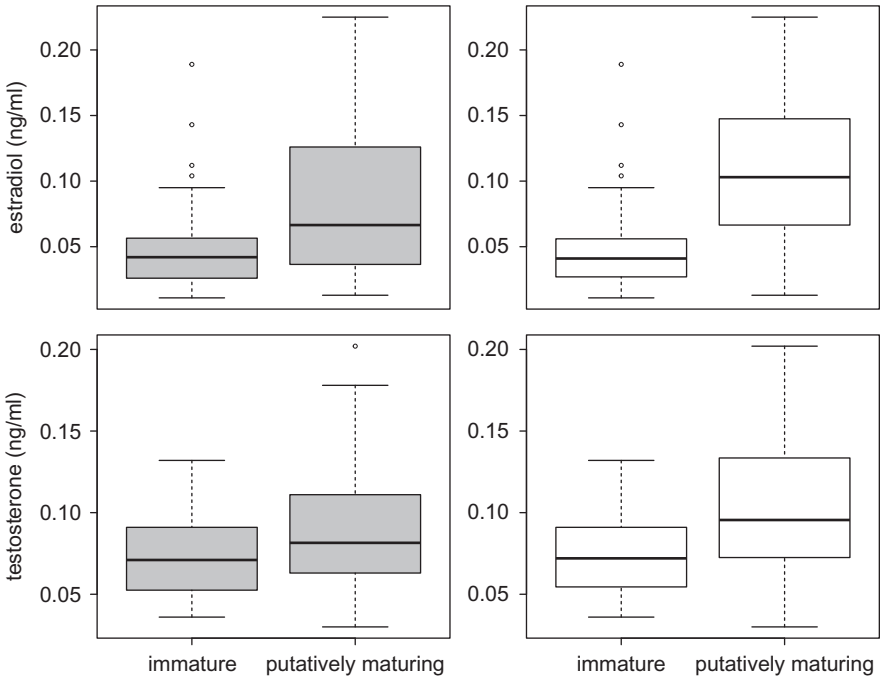

Figure 3: Plasma concentrations of estradiol and testosterone in immature and putatively maturing female southern flounder (n = 103). Plots on the left represent maturity staging based on the conventional oocyte staging criteria, and plots on the right are classifications based on modified oocyte staging (i.e. late cortical alveolus criteria). Box plot definitions are presented in Fig. 2.

Table 2: Spearman's ρ for gonadosomatic index, hepatosomatic index, estradiol and testosterone data collected in female southern flounder

	Gonadosomatic index	Hepatosomatic index	Estradiol	Testosterone
Gonadosomatic index	–	0.49	0.37	0.08
Hepatosomatic index	–	–	0.56	0.31
Estradiol	–	–	–	0.59
Testosterone	–	–	–	–

The best-performing models that did not include hormone data reclassified maturity successfully ~82% of the time. The inclusion of hormone data improved the reclassification success rate of the best-performing models to 87% (Table 3). In the best-performing hormone and non-hormone models, both TL and weight were included as predictors. In general, plasma E_2 concentration was a better indicator of maturity than plasma T concentration.

Lipid content and composition

In total, 125 individuals sampled in 2012 were selected for liver lipid content. Owing to analytical constraints, 39 of those fish were used for lipid classification analysis. Fish used for liver lipid content ranged in length from 235 to 550 mm TL (mean TL = 398 mm, SD = 75 mm) and in mass from 140 to 2560 g (mean = 880 g, SD = 513 g). Fish used for lipid classification ranged in length from 235 to 550 mm TL (mean TL = 401 mm, SD = 86 mm) and mass from 140 to 2560 g (mean = 953 g, SD = 616 g). Liver lipid content demonstrated a strong positive association with fish body size (Spearman's ρ = 0.78), with smaller fish nearly always having lower amounts of lipid than larger fish. During the summer period, liver lipid content increased linearly with fish body size, although we were unable to sample larger body sizes that were available in the autumn (as a result of large adults returning to estuaries and different gear types that target them). During the autumn, southern flounder also demonstrated an increase in liver lipid content at larger body sizes. The segmented regression had a lower Akaike information criterion (AIC) score than the linear model (425 compared with 449), with the breakpoint estimated to be 1101 g (SE = 116 g). The slope prior to the breakpoint was estimated to be 0.025 and the slope after the breakpoint 0.001. As expected, the pre-breakpoint slope was significantly different from 0, whereas the post-breakpoint slope was not

Table 3: Reclassification success rates (based on 10 000 randomizations) of several non-lethal models used to predict maturity in southern flounder

	Model	Reclassification success (%)	SD (%)
Non-hormone	TL + W	81.70	7.53
	TL + W + DOC	81.62	7.52
	W + DOC	79.44	8.16
	W	79.10	7.88
	TL	76.70	8.08
	TL + DOC	76.36	9.12
Hormone	TL + W + E_2	87.02	6.72
	TL + W + DOC + E_2	86.82	6.89
	E_2 + W	84.57	6.81
	E_2	84.08	6.86
	TL + W + T	83.95	7.07
	E_2 + DOC	83.81	7.16
	TL + W + DOC + T	83.54	7.37
	E_2 + T	83.26	7.32
	E_2 + TL	82.90	7.25
	T + W	81.52	7.46
	T + TL	79.12	8.37
	T	77.97	8.10
	T + DOC	76.81	9.08

The top tier of models are those without any hormone data included, whereas the bottom tier includes combinations of estradiol and/or testosterone data. Abbreviations: DOC, date of capture; E_2, estradiol (in picograms per millilitre); T, testosterone (in nanograms per millilitre); TL, total length (in millimetres); and *W*, weight (in grams).

(*P*-values are not calculated for coefficients, so significance was interpreted based on the confidence intervals for the coefficients overlapping zero, with overlap suggesting non-significance; Fig. 4).

In the liver tissue, TAG levels were high and PL levels low in larger individuals. It is possible that these larger fish were maturing; however, because our maturity model was calibrated with fish sampled in the autumn, we did not estimate maturity predictions for lipid classification samples as some of these fish were sampled in the summer. Both PL and TAG appeared to reach stable levels at a body mass >1 kg. Maturity predictions were closely aligned with stable PL and TAG levels in that most fish >1 kg had high levels of TAG and were predicted to be maturing. In contrast, smaller, putatively immature fish had high levels of PL and low levels of TAG. Furthermore, individuals with lower total lipid content had lipids consisting mainly of PL, with only a low amount of TAG, whereas the lipids of individuals with higher total lipid content consisted primarily of TAG (Fig. 5).

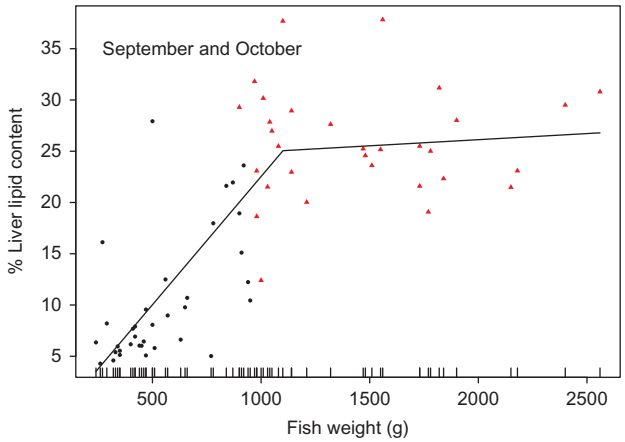

Figure 4: Liver lipid content (as a percentage) as a function of body weight in female southern flounder collected during autumn. The linear increase in liver lipid content (as a percentage) with increase in fish weight (in grams) plateaus at ~1101 g, as estimated by a segmented regression. Predicted maturity status was based on a model using individual length and weight. Black dots indicate those fish that are predicted to be immature, and red triangles indicate those that are predicted to be maturing. The two predicted maturity designations appear to have strong correlation with two different slope estimates. Tick marks along the *x*-axis represent individuals.

The predictive model used on autumn fish classified most individuals with >20% total lipid content as maturing.

Discussion

Blood hormone concentrations and associated reproductive state

Hormonal changes have been described in relationship to oocyte development in a number of fish species (e.g. Sol *et al.*, 1998; Pavlidis *et al.*, 2000; Pinillos *et al.*, 2003); however, fewer studies have examined differences in hormone concentrations between immature and maturing fish, and specifically using the formation of the zona radiata as the defining characteristic for the onset of maturity. In the present study, southern flounder that were placed in the cortical alveolus stage based on gonad histology were further categorized as either early or late cortical alveolus stage, which resulted in better separation between immature and maturing fish. Indeed, using the modified criteria, differences in sex steroid concentrations between immature and putatively maturing fish were more pronounced, providing greater separation between the two groups. This improvement was anticipated because of the close relationship between gonadal maturation and the concentrations of sex steroids in the blood plasma (Merson *et al.*, 2000; Dahle *et al.*, 2003). Similar to previously studied teleosts (*Rhombosolea tapirina*, Barnett and Pankhurst, 1999; *Gadus morhua*, Dahle *et al.*, 2003), an increase in E_2 was observed in putatively maturing females as ovarian development progressed. Overall, putatively

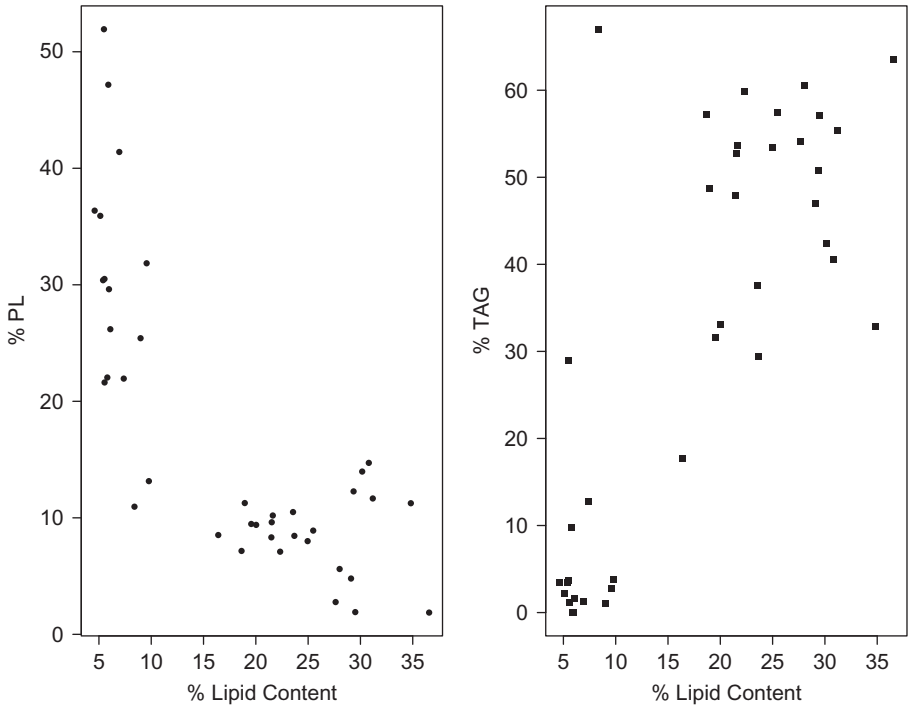

Figure 5: Percentage of phospholipids (% PL; left panel) and percentage of triacylglycerol (% TAG; right panel) as a function of liver lipid content (as a percentage) in $n = 66$ female southern flounder. Individuals with low liver lipid content contained low levels of TAG and high levels of PL, whereas individuals with high total lipid content contained high levels of TAG and low levels of PL.

maturing females in the present study had significantly higher sex steroid concentrations, an indication that individuals were preparing to participate in the upcoming spawning period. As described previously, E_2 is important to gonadal maturation because it triggers the production of VTG in the liver (Patiño and Sullivan, 2002), through the conversion of TAG to free fatty acids and, finally, to VTG (Wiegand, 1996).

The temporal patterns in GSI, HSI, T and E_2 each showed decreases before December, suggesting that many maturing females had already emigrated offshore, resulting in the capture of mainly immature females in late autumn. As further support of this notion, we captured only two fish older than 1 year during the months of November and December, suggesting that most older fish with a higher likelihood of being mature had already departed estuarine waters.

Patterns of lipid storage and composition

Lipid accumulation in the liver is important for the production of VTG in fishes, which is necessary for the growth and development of oocytes and embryos (Wiegand, 1996). In the present study, an increase in total lipid content in the liver with body size was observed in southern flounder until early autumn (September and October), when individuals began to show evidence of lipid mobilization. The observed pattern of greater lipid content with increased body weight (and expected maturity) is indicative of the storage of lipids in preparation for migration and oocyte development (e.g. Malavasi *et al.*, 2004; Jørgensen and Fiksen, 2006). During the late stages of maturation, lipids are incorporated into VTG and other lipoproteins that are necessary for reproductive development (Johnson, 2009), resulting in a decrease in total lipid content in the liver, similar to the pattern of decreased fats observed during spawning in Atlantic cod (Kjesbu *et al.*, 1991). Given that southern flounder emigrate offshore over a period of several months and our sampling did not extend beyond December or to offshore waters, we were not able to characterize the changes in liver lipids that would be associated with spawning-capable and regressing stages. We did, however, observe that total lipid content in the liver appeared to plateau during the autumn for even the largest fish. In addition, other indicators of maturation (e.g. plasma steroid concentrations, GSI) showed declining patterns during late autumn, perhaps suggesting that many of the fish collected during this period were not maturing in the current season and would have remained inshore rather than emigrate to spawn offshore.

The seasonal contribution of different lipid classes varied considerably between putatively maturing and immature fish in the present study. For larger, putatively maturing fish, the high levels of TAG and low levels of PL probably reflected the importance of storage lipids for migration and reproduction. Interestingly, TAGs were not only high in the autumn (October and November), but also in several fish sampled

in mid-summer (July). Most of these individuals were large and (based on our model using TL and *W*) predicted to be maturing, providing evidence that individual 'decisions' regarding maturation could occur several (≥6) months prior to any spawning activity. This information has the potential to inform our understanding of when decisions to initiate maturation occur in female southern flounder and may also shift the idea of what constitutes a maturing fish. Smaller, putatively immature fish contained low levels of TAG and high levels of PL, suggesting that the storage of TAG is unnecessary when an individual will not spawn in the upcoming winter and that excess energy is instead used for growth. Similar to patterns of TAG and PL in mature and immature fish, individuals with low and high total lipid content contained corresponding levels of lipid classes. Individuals with low total lipid content contained more PL and less TAG, and vice versa.

Several factors impact the ability of a fish to sequester lipids, including food availability, age, body size and state of the environment (Marshall *et al.*, 1999; Jørgensen and Fiksen, 2006), which can in turn affect the schedule of maturity and reproductive success (Shearer and Swanson, 2000). The relationship between lipid accumulation and successful reproduction has been documented on multiple occasions (e.g. Marshall *et al.*, 1999; Malavasi *et al.*, 2004; Jørgensen and Fiksen, 2006; Johnson, 2009) and should be used more routinely to supplement more traditional metrics of reproductive output (e.g. spawner abundance, fecundity) in fisheries assessment and management.

Conclusions

The management of exploited fishes may be enhanced through improved understanding of how and why a species 'decides' to reproduce. Typically, size- or age-based fisheries regulations meant to protect immature females are based solely on the macroscopic examination of gonads, and resulting maturity schedules also do not account for temporal variation in environmental conditions that can impact stock reproductive output, such as changes in temperature and earlier maturation (Atkinson, 1994). Often, total spawning biomass and/or egg production is overestimated, leading to overfishing (Jørgensen and Fiksen, 2006). For instance, Kjesbu *et al.* (1991) reported that the real fecundity of *G. morhua* was 20–80% lower than potential fecundity owing to the nutritional status of the stock. Currently, physiological and endocrine markers of fish condition and/or state are not routinely applied in fisheries management; however, with more focused research these important aspects of fish life-history strategies can contribute to management objectives. Some physiological methods to assess fish condition or reproductive status also provide the opportunity for non-lethal sampling, which may be important for threatened species (e.g. Heise *et al.*, 2009). Size-based methods (e.g. TL or GSI) may still be the single most informative maturity indicator, but for species that mature over a wide range of sizes or for which continuous sampling is not available, physiological indicators show promise toward refining spatiotemporal maturity estimates.

Advancing our understanding of physiological states and the factors that influence them helps to improve our understanding not only of a species' life history, but also of the environmental drivers and how the interaction of physiology and environment manifest in somatic growth and reproductive potential. We know that most species do not adhere to simple and invariant schedules of maturation, and the growing identification of important physiological information helps both to describe the variability we observe and to provide information to managers tasked with conserving individuals and biomass. The reproductive biology of southern flounder is mainly evaluated using macroscopic techniques during the emigration period, yet patterns in lipid storage and composition provide evidence that southern flounder initiate maturation several months prior to spawning. In general, hormone and lipid investigations, such as the present study, have the potential to bring to light valuable information regarding the complex sequence of physiological and biological changes that result in maturation and subsequent spawning success.

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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