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Seasonal and spatial changes in sex hormone levels and oocyte development of bonefish (*Albula vulpes*)

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Abstract Bonefish (*Albula vulpes*) support an economically important fishery, yet little is known regarding the reproductive biology of this species. Blood and oocyte samples were collected from wild female bonefish (*Albula vulpes*) during (February and April, 2017) and outside (September, 2017) the spawning season in Grand Bahama Island, The Bahamas. Fish reproductive state was evaluated using histological analysis of the oocytes and determination of sex hormone levels of 17β-estradiol and testosterone in the plasma. The presence of three different cohorts of oocytes in bonefish females suggests group-synchronous ovarian development. Levels of 17β-estradiol were low in individuals sampled outside of the spawning season relative to fish sampled during spawning months. Testosterone levels did not change as female bonefish entered the spawning season. Within the spawning season, bonefish are commonly found along shallow water flats, or in pre-spawn aggregations (PSA). The diameters of late vitellogenic oocytes collected from PSA fish were significantly larger than those from the flats fish. Levels of 17β-estradiol did not differ between PSA and flats fish; however, testosterone levels were significantly higher in fish from the PSA. These results indicate that as bonefish are transitioning to the PSA from flats habitats, vitellogenesis is still occurring. However, when and where final maturation commences in reproductively active bonefish remains unclear.

Keywords Bonefish · Reproductive development · Sex hormone · Oocyte

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Introduction

Bonefishes (*Albula* spp.) occur in shallow waters throughout the tropical regions of the world (Crabtree et al. 1996; Johannes and Yeeting 2000; Murchie et al. 2010). Within the subtropical to tropical waters of the northwest Atlantic, including the Florida Keys (Florida, USA), Bahamas, and Caribbean Sea (Hildebrand 1963), *Albula vulpes* is the most common species present and supports an economically important recreational fishery (Fedler 2010; Fedler 2013; Wallace and Tringali 2016). Within these regions, *A. vulpes* (hereafter "bonefish") commonly inhabit interconnected, nearshore, shallow (<2 m) tidal flats, which include mangrove forests, seagrass and macroalgal beds, sand shoals, and occasionally patch reefs (Murchie et al. 2013). The variability in flats habitat provides bonefish diverse foraging opportunities for prey items such as crabs, fishes, bivalves, polychaetes, and small crustaceans (Colton and

Alevizon 1983; Crabtree et al. 1998; Layman and Silliman 2002). From October to May, bonefish migrate from flats to aggregation sites in many of these regions (Danylchuk et al. 2011; Boucek et al. 2018; Adams et al. 2018). Based on the presence of developed gonads and increases in lipid storage around the liver and gonads in individuals at both flats and PSAs, this aggregating behavior and habitat selection likely play a critical role in the reproductive strategy of bonefish (Murchie et al. 2010; Danylchuk et al. 2011).

Although pre-spawn aggregation (PSA) sites are still being identified for bonefish, they can generally be characterized as deep (>4 m), protected nearshore basins, in close proximity to both flats habitat and the continental shelf (Adams et al. 2018**)**. Movement to these sites occurs largely with lunar periodicity (full and new moon) as schools of fish (~1000 or more individuals) assemble at the PSA site, while non-spawners remain on the flats (Danylchuk et al. 2011). It is important to note that bonefish are not commonly observed at PSA sites outside of the spawning season (J. Lewis, pers. obs.).

Neither flats nor PSAs are thought to act as spawning grounds for bonefish. This is based on the general absence of fully mature and/or hydrated oocytes typically associated with imminent spawning at these sites (Crabtree et al. 1997; Larkin 2011), and no observations of gamete release in the PSAs (Danylchuk et al. 2011; Danylchuk et al. 2018; Adams et al. 2018). Observational and acoustic tracking data also indicate fish migrate offshore to spawn, possibly off the continental shelf during the late-evening during the full moon of the reproductive months (Crabtree et al. 1997; Danylchuk et al. 2011). Therefore, the role that flats and PSA habitats play in facilitating the reproductive development of bonefish prior to spawning remains unclear.

It is widely accepted that oocyte development in vertebrates during spawning is heavily regulated by a cascade of hormones along the hypothalamus-pituitary-gonadal (HPG) axis (Sower et al. 2009). Along this axis, gonadotropins released from the pituitary stimulate the synthesis and secretion of critical reproductive hormones by the gonads. Cyclical changes in the occurrence and concentrations of reproductive hormones are widely known to occur in association with both reproductive behavior and gonadal development. In females, oocyte development (oogenesis) occurs as germ cells develop into oogonia and are eventually released as ova during spawning (Lubzen et al. 2010). The reproductive hormones primarily responsible for the growth of oocytes during early and advanced oogenesis are 17β-estradiol and testosterone (Nagahama and Yamashita 2008).

During this time, vitellogenin synthesis is stimulated in the liver, facilitating the uptake of yolk protein within the oocyte and promoting substantial oocyte growth prior to final maturation (Lubzen et al. 2010). However, this process can vary greatly by fish species and depends on reproductive strategy, synchrony of oocyte development, and spawning frequency (Rocha and Rocha 2006).

To date, no studies have assessed bonefish reproductive sex hormone and gonadal development. Therefore, the objectives of this study were to: (1) provide the first reproductive profile in wild bonefish via simultaneous analysis of ovary development and plasma sex hormone levels (17β-estradiol and testosterone), and (2) describe differences in the reproductive status of fish at flats versus PSA habitat during the spawning season. This work was conducted in the relatively pristine central and eastern end of Grand Bahama Island, The Bahamas, where substantial flats habitat exists and a known PSA has been identified. Based on our current understanding of the spawning strategy of migratory fishes, elevated levels of both testosterone and 17β-estradiol were expected for sexually mature females sampled during the spawning season (Ueda et al. 1984; Barannikova et al. 2004). Also, since PSAs are thought to act as staging areas for spawning capable fish, more progressed oocyte development were expected from these individuals relative to those collected from the flats. Through examination of hormone concentrations in concert with gonadal histology, high-resolution data regarding the reproductive status were obtained.

Methods

Initial sample collection and preservation

Female bonefish were opportunistically sampled from several locations along the southern shore of Grand Bahama Island, The Bahamas (Fig. 1), during the day (08:00 to 17:00) of the full moon for February, March, April, and September 2017. Samples collected during September 2017 were considered to be from non-spawning fish (Danylchuk et al. 2011). Fish were

collected from two general sites: 1) a pre-spawn aggregation located in water greater than 4 m deep along the southeast coast of the island ($n = 1$ site) and 2) shallow tidal flats less than 1 m deep ($n = 6$ sites), which are not used as a PSA site (Fig. 1). Fish in the PSA were captured via hook and line using cut shrimp or white jigs and 50 lb. test line. Fish fight time did not exceed 2 min. Individuals on the tidal flats were captured using a 50 × 1 m beach seine with a 2.5 cm mesh. All individuals collected were kept for 3–5 min in plastic, floating containers modified with holes to allow adequate water exchange. During sampling, fish were held inverted to induce a state of tonic immobility. Bonefish are not sexually dimorphic so fish that did not readily release gametes (spawning-ready males frequently release sperm when abdominal pressure is applied) were cannulated for oocytes using a soft-tube catheter (Bard 100% latex-free infant feeding tube, 8Fr (2.27 mm diameter, 26 cm length) attached to a 3 ml syringe barrel. This method was adapted from Rottmann et al. (1991). Captured females were first measured for fork length (FL) and cannulated oocytes (volume of 1–2 ml) were expelled into a 2.0 ml capped vial filled with 1 ml of 10% neutral buffered formalin. During the September sampling, only bonefish that produced identifiable ovarian tissue during cannulation were identified as females. Following oocyte collection, blood was then drawn from the ventral side of the fish's caudal vein using a heparinized syringe and deposited into a lithium heparin lined BD vacutainer™. Blood samples were placed in a cooler above wet ice for later processing. At the laboratory, plasma was separated from blood by centrifugation (2500 rpm for 20 min) and stored at −80 °C until specific assays could be performed.

In total, blood samples were collected from 37 female bonefish (456–649 mm FL) at the flats ($n = 27$) and PSA ($n = 10$) sites during the full moons of February 2017 ($n = 13$) and April 2017 ($n = 22$), as well as two samples collected from a PSA during the full moon of

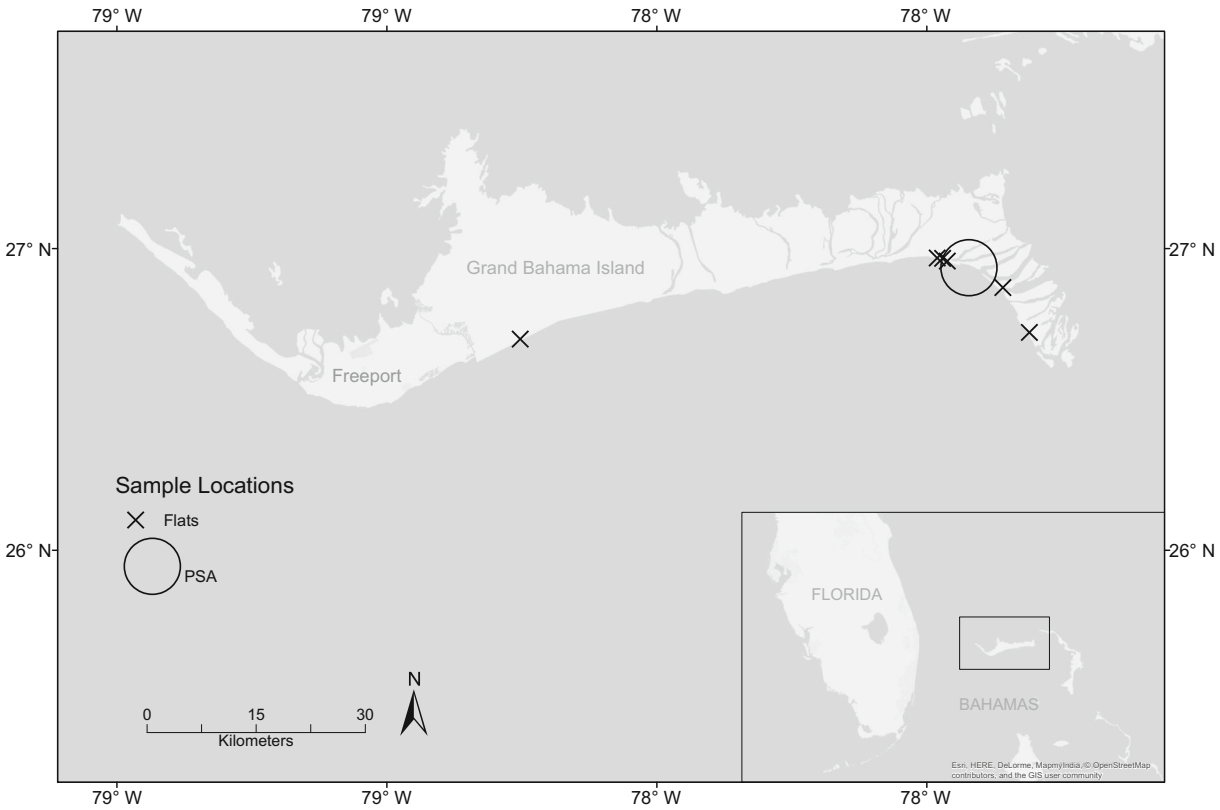

Fig. 1 Map showing sampling locations for both flats and pre-spawn aggregation (PSA) sites during spawning (February, March, and April 2017) and non-spawning (September 2017) months of bonefish (*Albula vulpes*) in Grand Bahama Island, Bahamas. Exact PSA location has been generalized for conservation purposes (Adams et al. 2018)

March 2017. Blood samples were also collected from female bonefish at several flats habitats in a non-spawning month (September 2017, $n = 12$). Oocytes from 20 bonefish females were collected and histologically prepared from flats sites, and seven from the PSA sites during the full-moon of April 2017. Bonefish from the PSA was not sampled for either blood or eggs during February due to logistical difficulties that limited our ability to target the deeper water fish.

Histological preparation of oocytes

Oocytes stored in 10% neutral buffered formalin during field collection were transferred to 70% ethanol prior to preparation (Barber 1996; Wilson et al. 2005). Oocytes were dehydrated through a series of ethanol solutions (70–100%) for 60 min, clarified in toluene, and embedded within paraffin wax. A microtome cut 8–10 μm thick sections from the embedded samples, which were stained with hematoxylin and eosin before being mounted on pre-labeled glass slides for examination. A subset of oocytes ($n \geq 60$ oocytes) from each female was photographed from each histology sample using an OLYMPUS SZX7 stereozoom microscope at magnifications of 30–200×. For each subset, developmental stages of observed oocytes were categorized as late vitellogenic (LV), cortical alveolus (CA), and primary growth (PG) based on the classification scheme developed by Crabtree et al. (1997) (Fig. 2). In general, PG oocytes occur as the initial development phase and develop into CA oocytes during early reproductive development. The final stage of oocyte development prior to final maturation is LV and exhibit both substantial lipid accumulation and a relatively large size (Brown-Peterson et al. 2011). If fully mature (hydrated) oocytes were present, they were also categorized and enumerated. Oocytes in the LV stage were measured for diameters (μm) by bisecting the nucleus through the center of the oocyte using a calibrated ruler tool within the image processing software (West 1990). Only LV oocytes fully enclosed within the frame were included. In total, 2228 oocytes were categorized, and 1775 LV oocytes were measured.

Sex-hormone plasma level determination

17β-estradiol and testosterone concentration levels were quantified via enzyme-linked immunoassay (ELISA) kits (Cayman Chemical Company, USA). For extraction, a 100 μl plasma sample was diluted with ELISA buffer based on the manufacturer specifications (Cayman Chemical Company, USA). Hormones were then extracted twice by vigorous vortex using dichloromethane for 17β-estradiol and diethyl ether for testosterone. The supernatant organic phase was removed via Pasteur pipette, evaporated under a gentle stream of nitrogen at 30 °C, and reconstituted using ELISA buffer prior to plating. This process was repeated according to the protocol provided by Cayman Chemical Company, USA. Samples were run at two dilutions to fulfill manufacturer requirements and minimize interference within wells. A control sample provided by the manufacturer was serially diluted according to manufacturer specifications and run as a standard with each plate of samples. Plates were analyzed via absorbance at a wavelength of 405 nm using a microplate reader (Biotek, Synergy H1, USA). Absorbance values were converted to concentration values ($pg.ml^{-1}$) using software provided by Cayman Chemical Company, USA.

Statistical analysis

Reproductive development was compared between sites (Flat vs. PSA) using mean oocyte diameter as a response variable (West 1990). This analysis was conducted using a non-parametric Kruskal Wallis test, since parametric assumptions of normality and homoscedasticity could not be met. Analyses were performed in R (R Core Team 2014).

Differences in the frequency of occurrence of developmental stages (PG, CA, and LV) at PSA and flats sites were assessed using a permutational multivariate analysis of variance (PERMANOVA with 999 permutations), including a posteriori pair-wise comparisons with PRIMER 7 (v. 7.1.12) and PERMANOVA+ (v.1.0.2). Assumptions of homoscedasticity were verified with a PERMDISP test, and data were transformed (log or arcsine square root) when necessary.

Differences in sex hormone (17ß-estradiol and testosterone) concentrations between sampling months were analyzed using a one-way ANOVA for each hormone. Tests were conducted following a square-root transformation of data to meet parametric assumptions of normality and homoscedasticity. Comparison by month included only bonefish females sampled on the flats in February, April, and September 2017. Differences in sex hormone concentrations during the spawning season were analyzed across sites (Flat and

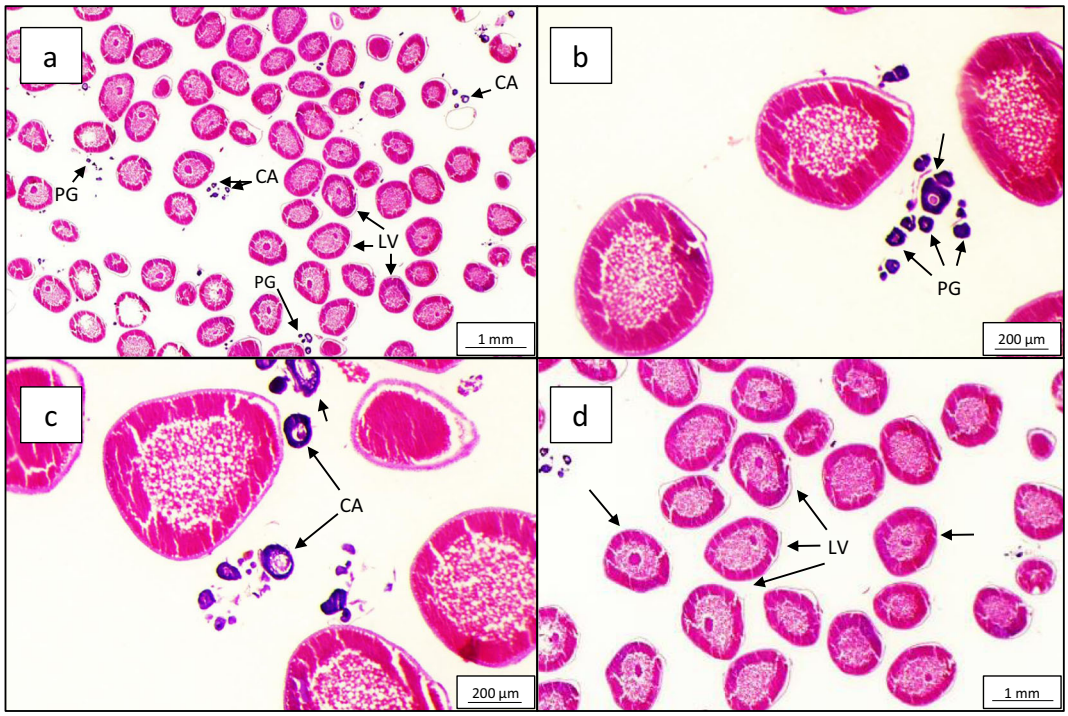

Fig. 2 Micrographs of histologically sectioned oocytes sample collected from a wild female bonefish (*Albula vulpes*) on a shallow water flat during full moon (April 2017). **a** three different developmental oocyte stages are present: **b** primary growth (PG), **c** cortical alveolar (CA), and **d** late vitellogenic (LV). Oocyte samples collected at the pre-spawn aggregation (PSA) were visually similar to flats samples

PSA) using a two-sample T-test and specifically used samples collected during spawning months (February, March, and April 2017).

Results

Histology

The diameter of late-vitellogenic oocytes sampled at the PSA sites during April 2017 (591 ± 134 μm) were significantly larger than those from the flats during the same period (556 ± 129 μm) ($H = 345.21$, $p < 0.001$). The frequency of LV oocytes ranged from a mean of 63% (±15%) in fish taken on the flats to a mean of 78% (±7%) in fish collected from the PSA. The overall frequency distribution of oocyte stages was significantly different between flats and PSA sites (*Pseudo* $F_{(1, 24)} = 4.97$, $p < 0.05$; Fig. 3). An average dissimilarity of 12.9% existed between samples collected from these two sites and was driven by the relative percentages of oocytes at LV and PG stages. The occurrence of LV stages were higher in bonefish from PSA than the flats and contributed up to 41.5% of the dissimilarity. The occurrence of oocytes at PG stages was lower in bonefish from the PSA than the flats and contributed up to 36.4% of the dissimilarity. No hydrated oocytes were observed in any fish sampled regardless of capture site.

Sex hormone concentration

Seasonal differences

Mean 17ß-estradiol levels of 3000–4000 pg·ml^{-1} were detected during the spawning months of February and April combined, dropping to <1000 pg·ml^{-1} in the non-spawning month of September (Fig. 4). These levels were significantly different among seasons ($F_{(2, 35)} = 12.920$, $p = <0.001$). A post-hoc pairwise comparison (Tukey) indicated that this difference was driven by significantly higher levels of 17ß-estradiol during the reproductive season (February: $q = 5.668$, $df = 1$, $p = <0.05$; April: $q = 6.721$, $df = 1$, $p = <0.05$) compared to the non-reproductive season (September). 17β-estradiol levels did not significantly differ between February and April sampled bonefish ($q = 4.247$, $df = 1$, $p = 0.825$).

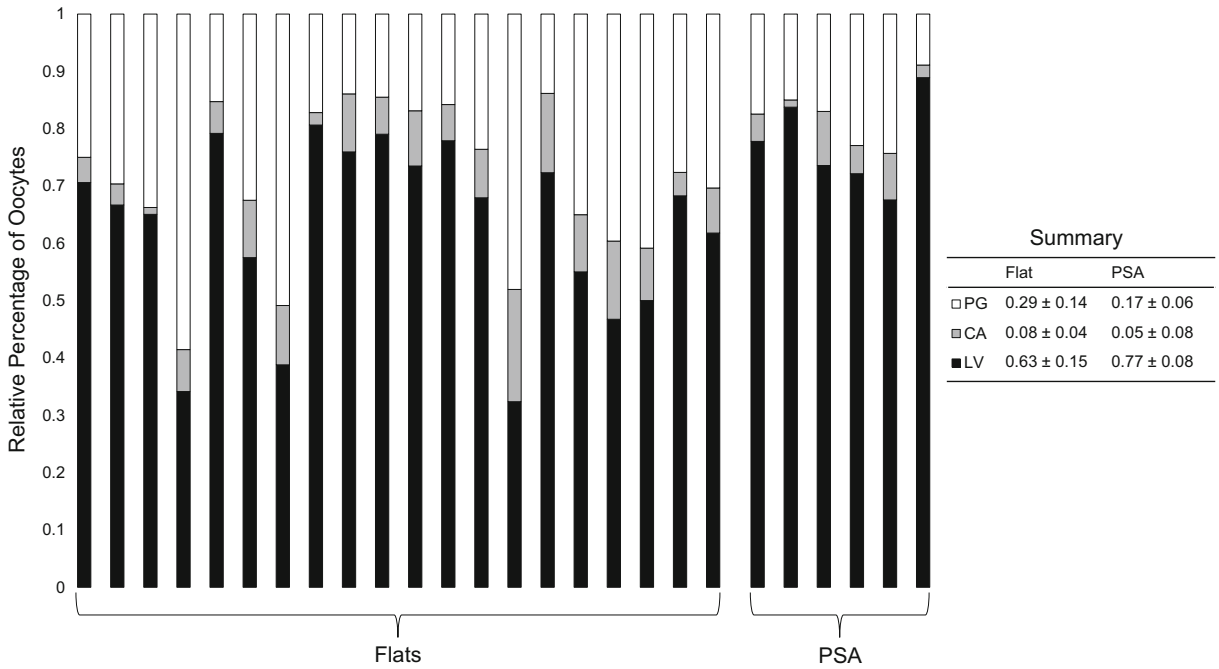

Fig. 3 Proportional occurrence for each of the three oocyte stages: late vitellogenic (LV), cortical alveolar (CA), primary growth (PG) at the two sites (flats and pre-spawn aggregation [PSA]) sampled during the spawning month of April 2017. Oocytes were collected from sexually mature bonefish (*Albula vulpes*) females. Each bar indicates an individual fish. Table displaying mean ± SD by oocyte stage has also been included

Mean testosterone levels of 2000–3000 pg.ml^{-1} were not significantly different between females sampled during February, April, and September ($F_{(2,\ 32)} = 1.947$, $p = 0.160$) (Fig. 4).

Flats vs. PSA

Within the spawning season, mean 17ß-estradiol levels at the PSA were not statistically different from fish

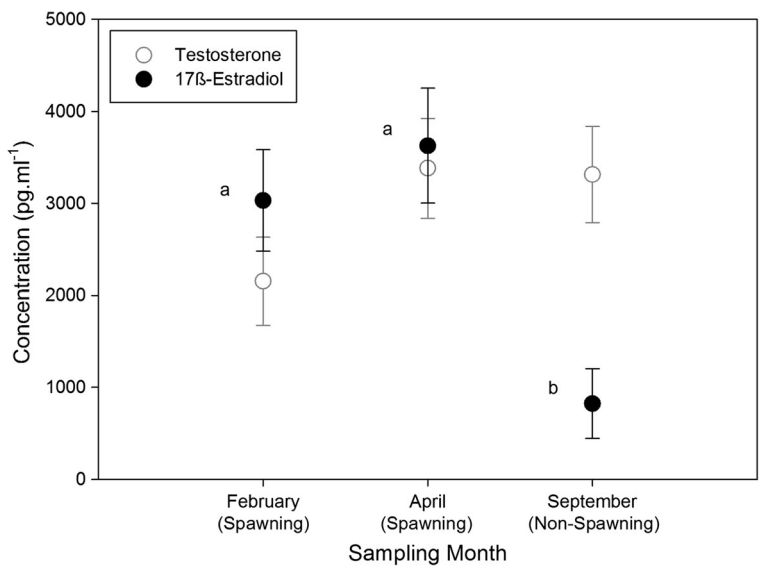

Fig. 4 Monthly plasma concentrations of 17β–estradiol and Testosterone (mean ± SEM) from bonefish (*Albula vulpes*) females sampled in the flats during spawning (February and April 2017) and non-spawning (September 2017) months (mean ± SEM). Letters indicate significant differences in levels of 17β –estradiol between seasons

sampled on the flats ($t = -1.632$, $df = 34$, $p = 0.112$; Fig. 5). Testosterone levels were significantly higher in bonefish from PSA when compared to the fish sampled from flats ($t = -5.021$, $df = 32$, $p = <0.001$; Fig. 5).

Discussion

This study revealed spatial variation in levels of testosterone in female bonefish from flats and pre-spawn aggregation locations. Furthermore, differences in 17ß-estradiol and testosterone levels between in-season and out-of-season bonefish females provide information regarding the spawning preparation of bonefish as they enter the reproductive season. The physiological significance of these results is examined below.

Seasonal differences

The increased levels of 17ß-estradiol in bonefish females sampled along the flats during the spawning season compared to the non-spawning season follow a trend seen in other marine teleosts as fish begin the process of vitellogenesis (Scott et al. 1983b; Taghizadeh et al. 2013; Zupa et al. 2017). Increased plasma concentrations of 17ß-estradiol facilitate rapid oocyte growth through vitellogenesis as vitellogenin, a hepatically derived plasma precursor, is sequestered as yolk protein within the developing oocyte (Lubzen et al. 2010). In doing so, oocytes increase in size and nutritive quality, eventually reaching a developmental state when final enlargement and maturation through hydration occur (Wallace and Selman 1981). The significantly elevated levels of 17ß-estradiol found in sampled bonefish during February and April corresponds with previous findings of spawning behavior occurring in female bonefish around the full moons from October to May (Danylchuk et al. 2011).

Bonefish sampled both prior to, and during the spawning season showed high levels in testosterone. In many spawning teleost fishes, testosterone is secreted around the same time as 17ß-estradiol (Scott et al. 1983a; Mandich et al. 2004; Zupa et al. 2017) and is thought to fulfill two major roles: the regulation of gonadotropin secretion by the pituitary (Scott et al. 1980; Bommelaer et al. 1981) and/or serve as the androgenic precursor in the formation of estrogens via an aromatase enzyme (Lambert et al. 1971; Scott et al. 1980; Kagawa 2013). Our results indicate that gonadal production of testosterone occurs prior to 17ß-estradiol.

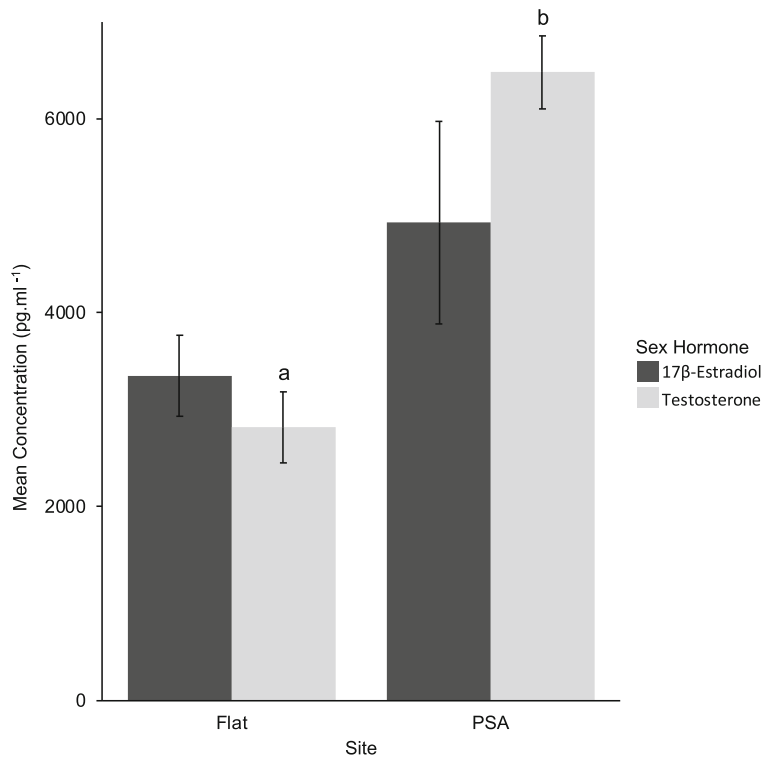

Fig. 5 Plasma concentrations of 17β –estradiol and testosterone (mean ± SEM) at flats and pre-spawn aggregation (PSA) sites in female bonefish (*Albula vulpes*) sampled during three spawning months (February, March, and April 2017). Letters indicate significant differences in Testosterone levels between sites

The sampling month (September) for the non-spawning season occurred roughly 1 month before the winter spawning season historically begins (October) (Danylchuk et al. 2011) and may indicate that, just prior to vitellogenesis, bonefish increase testosterone production in preparation for synthesis of 17ß-estradiol. Future sampling should take place at the end of the spawning season to assess testosterone concentrations as bonefish proceed into non-spawning season.

Flats vs. pre-spawn aggregation

The high mean level of 17ß-estradiol found in bonefish females sampled on the flats during the spawning season relative to fish sampled in September (outside of the spawning season) indicates that vitellogenesis begins at these habitats before bonefish start their migration to the PSA sites. We expected to see slightly lower levels of 17ß-estradiol in fish sampled on flats, based on the high proportional occurrence of early development stage oocytes (PG and CA) in flats sampled fish compared to those at the PSA. Visibly, mean concentration values of 17ß-estradiol seem lower in flats sampled fish compared to PSA fish and correlates well with the smaller diameters of late vitellogenic oocytes found in flats fish. However, statistically the differences in 17ß-estradiol between these two sites was insignificant, indicating that while levels may subtly differ by site, vitellogenic development is continuing while fish migrate too and aggregate at PSAs. In a study conducted on chum salmon (*Oncorhynchus keta*), levels of 17ß-estradiol found in females offshore (~15 ng/ml) were statistically similar to those at the pre-spawn staging sites in river mouths (~14 ng/ml) (Ueda et al. 1984), indicating that vitellogenesis had also not yet ceased. Similarly, our findings suggest that vitellogenesis continues to take place at the PSA, likely up until the moment bonefish move offshore to spawn. Generally, 17ß-estradiol values at the individual level are expected to remain elevated, falling once fish have completed vitellogenesis (Stuart-Kregor et al. 1981; Barannikova et al. 2004). However, in this study we did not sample any fish with hormone levels indicating they had reached the end of vitellogenesis or recently spawned. Therefore, it was not possible to determine whether 17ß-estradiol typically dissipates following spawning or remains elevated in preparation for the next spawning event. Future analyses that incorporate post spawning fish hormone levels would be beneficial to our understanding of 17ß-estradiol dynamics in spawning female bonefish.

Comparisons of bonefish oocyte development between sites further supports the hypothesis that vitellogenic development is still occurring at the PSA. Bonefish sampled on the flats exhibited varied compositions of oocytes, likely a result of capturing both fish moving towards the PSA and those still preparing the next clutch. The measured increase in the occurrence of late vitellogenic oocytes, and conversely decreased occurrence of PG oocytes at the PSA compared to flats, suggests ovaries are more developed at the PSA. Late vitellogenic oocytes at the PSA were also larger than those from flats fish, which is an indication of further advanced development (West 1990). The slightly elevated mean levels of 17ß-estradiol levels found in PSA fish compared to those from flats could explain this difference.

The absence of fully mature oocytes from bonefish collected in any of the sampling events raises the question of temporal proximity of bonefish females to the completion of vitellogenesis (i.e., final maturation and hydration). In many studies, the completion of vitellogenesis is marked by a sharp decline in 17ß-estradiol levels and typically occurs with the onset of full oocyte maturation (Whitehead et al. 1978; Idler et al. 1981; Zupa et al. 2017). In this study, 17ß-estradiol levels were sustained in sampled female bonefish at the PSA, indicating vitellogenesis was still occurring. However, several studies have documented maximum levels of testosterone occurring at the end of vitellogenesis in female fish (Campbell et al. 1976; Ueda et al. 1984, Ijiri et al. 1995). These findings were explained by the occurrence of a holding phase, where testosterone continues to be released by the follicle after the aromatase enzyme is turned off (Kime 1998). Indeed, we have observed that while 17ß-estradiol levels seem to remain stable between the flats and PSA habitats, it is likely that vitellogenesis concludes at the PSA site based on the significant increase in testosterone levels observed in bonefish females at these sites. The continued production of testosterone might also suggest that this androgen could play a role in stimulating the synthesis of a maturation inducing hormone to cue hydration (Crim et al. 1981). For instance, increases in plasma gonadotropins that cue final oocyte development occurred in both female rainbow trout (*Salmo gairdneri*) and white suckers (*Catostomus commersoni*) after peaks in testosterone occurred (Scott et al. 1983a; Scott et al. 1983b).

Therefore, the increase in testosterone found at the PSA sites likely indicates that female bonefish are indeed close to initiating final maturation. Field observations from Danylchuk et al. (2011) suggest that this spawning migration takes place at night. Since sampling efforts had ceased at this point due to the inability to maintain visual contact with the aggregation, it is possible that full maturation may be occurring in the late evening at the PSA, during their journey offshore, or at the location where spawning occurs.

The hormonal levels of 17ß-estradiol and testosterone collected in this study provide baseline data regarding bonefish development useful for identifying the occurrence of reproductive dysfunction that may be occurring in highly impacted areas. For example, the bonefish population in the Florida Keys has declined in the last 20 years despite being a predominantly catch-and-release fishery (Larkin 2011; Santos et al. 2017; Rehage et al. 2018). The causes of this decline are likely complex given the series of anthropogenic disturbances within Florida Bay (USA) and the Florida Keys (Fourqurean and Robblee 1999; Larkin 2011; Santos et al. 2017). Pre-spawn aggregations have also not yet been documented in Florida, an interesting fact considering recently compiled traditional ecological knowledge indicates this spawning behavior is common globally where most *Albula* spp. exist (Johannes and Yeeting 2000; Adams et al. 2018). In regions where anthropogenic impact is still relatively low (i.e., outer Bahamian islands), habitat, diet, and movement characteristics of bonefish are very similar to what is found in South Florida (Larkin 2011). By sampling in these more remote areas, we have been able to provide baseline information regarding bonefish life history, particularly reproductive physiology, to aid our ability to identify and address factors influencing the survival of bonefish in impacted areas such as Florida.

Conclusion

Our findings highlight that vitellogenesis occurs at both sites, beginning at the flats and likely concludes at the PSA. The elevated testosterone levels prior to the spawning season are perhaps preparatory reserves for aromatase but may facilitate some other process not yet understood. During the spawning season, the absence of fully mature and hydrated oocytes at any sampling event indicates that final maturation is likely occurring at some point offshore at night, where spawning is presumed to take place. It is also possible that oocyte maturation occurs at night in the PSA when sampling was not taking place. However, given the fact that no sampled fish showed evidence of hydrated eggs, final maturation at the earliest occurs just prior to offshore migration. Regardless, given the short period of time schools spend offshore based on acoustic tracking (~8 h), maturation is occurring very quickly. Further research regarding this period of development is critical to our understanding of bonefish reproductive physiology.

Our understanding of bonefish reproductive development would benefit from a continued sampling approach to explore these metrics at an annual scale, which would include the fall and summer non-spawning months. Furthermore, because other PSAs continue to be identified throughout much of the geographic distribution of this genus, assessing the potential variability in reproductive importance of sites across different islands would also aid in our understanding of bonefish spawning biology. In general, much is still unknown regarding bonefish reproduction, including spawning locations and habitat characteristics, temporal and spatial spawning cues, and the frequencies with which individuals spawn. These data gaps must be addressed to sustain declining populations of this economically important species.

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