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Reproductive physiology of bonefishes (*Albula* spp.) across the Northwest Bahamas

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Abstract Bonefishes (*Albula* spp.) are classified within the superorder Elopomorpha, which is comprised of over 1000 species that share a unique leptocephalus larval stage. Bonefishes have a circum-tropical distribution, inhabiting inshore shallow water flats and gathering in presumptive nearshore pre-spawn aggregations (PSA) during spawning months. These fishes support economically important recreational fisheries and subsistence fisheries throughout their ranges, yet little is known regarding their reproductive biology. Analysis of oocyte development and nutrient composition, and sex and gonadotrophic hormone levels, was conducted on females sampled in Grand Bahama, Central Andros, and South Andros, The Bahamas, to assess their reproductive state. Fish collected from the flats habitats along all three islands exhibited four major reproductive phases (immature, developing, spawning capable, and regressing). In contrast, all females captured at presumptive PSA sites had eggs in the final stage of oocyte maturation, significantly higher levels of all reproductive hormones (17β-estradiol, testosterone, and LH), larger vitellogenic oocytes, and oocytes exhibiting germinal vesicle migration and germinal vesicle breakdown. In addition, monthly variability in hormone levels and spawning readiness between Grand Bahama and Andros PSAs suggest that peak spawning times may differ among regions. Fatty acid and free amino acid composition and profiles, with high proportions of docosahexaenoic acid, histidine, and taurine, suggest that these nutrients are not only relevant as energy reserves, but also help achieve buoyancy and osmoregulation of oocytes. This study expands upon our understanding of fish reproductive and developmental physiology, and indicates potential factors influencing the survival and recruitment of bonefishes.

Keywords Reproductive development · Aggregation · Reproductive hormones · Oocyte stage · Fatty acid · Amino acid

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Introduction

Many tropical marine fishes exhibit aggregating behavior for a variety of reasons including safety, food acquisition, migration, and/or spawning (Domeier 2012). Spawning aggregations have gathered considerable interest in both the scientific community and in fisheries management due to their importance in sustaining valuable fish stocks (Erisman et al. 2017). When fishes aggregate for spawning, they adaptively select for

specific habitats that maximize larval survival and subsequent recruitment (Johannes 1978). Many species congregate at pre-spawning aggregation (PSA) sites before undergoing a final migration as large schools to the actual spawning site. This behavior has been observed in both tarpon (*Megalops atlanticus*) (Crabtree et al. 1997) and bonefish (*Albula* spp) (Danylchuk et al. 2011, 2019 Adams et al. 2019a, b). Aggregation at a PSA may help synchronize gonadal maturation, ensure arrival at spawning locations at the same time, and provide protection in numbers from the assumed elevated predation risk (Domeier 2012). However, the amount of knowledge about spawning aggregations is limited to a relative handful of species that have received research attention because of their susceptibility to overharvest (Colin et al. 2003; Sadovy and Domeier 2005; Sadovy de Mitcheson et al. 2008).

Bonefishes (*Albula* spp.) comprise a single genus of circum-tropical-subtropical marine fishes that inhabit shallow habitats (Alexander 1961). In the Caribbean Sea and western North Atlantic Ocean, *Albula vulpes*, along with Atlantic tarpon (*Megalops atlanticus*) and Permit (*Trachinotus falcatus*), supports a significant recreational catch and release fishery (known as the *flats fishery*; Adams et al. 2019a, b) with an estimated annual economic impact of $465 million (USD) in the Florida Keys (Fedler 2013) and $50 million in Belize (Fedler 2014). In the Bahamas, the annual economic impact of the recreational bonefish fishery exceeds $141 million (Fedler 2010). Throughout the year, bonefish occur within shallow (< 2 m), nearshore coastal habitats comprised of mangroves, algae, sand and mud bottom, seagrass, and limestone outcroppings which support diverse communities of fishes and invertebrates. These habitats are commonly referred to as "flats" and provide a variety of resources for both resident and transient organisms (Murchie et al. 2013; Adams et al. 2019a, b). Much has been learned in recent years about bonefish biology, but considerable gaps remain. Mark-recapture data have showed that adult bonefish have relatively small home ranges (Boucek et al. 2019). Adults seasonally migrate from home ranges to pre-spawning locations in deeper waters (> 4 m) near both the continental shelf and adjacent flats. Bonefishes spawn at night, near full and new moons, between October and April (Adams et al. 2019a, b; Danylchuk et al. 2011).

To date, little research exists regarding the reproductive ecology of *A. vulpes* or the physiological role these aggregation events play in the spawning preparation process. Previous research conducted in the Bahamas indicates at least one PSA occurs at each of the islands assessed (Andros, Abaco, Grand Bahama, Long Island, and Eleuthera) (Adams et al. 2019a, b), and that oocyte development is more advanced and sex hormones are higher in females captured from PSAs than females captured on the flats (Mejri et al. 2019; Luck et al. 2019).

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 fishes. Oocyte development (oogenesis) occurs as germ cells develop into oogonia and are eventually released as ova during spawning (Lubzens et al. 2010). The reproductive hormones primarily responsible for the growth of oocytes during early and advanced oogenesis are luteinizing hormones (LH), 17β-estradiol, and testosterone (Nagahama and Yamashita 2008). During this time, vitellogenin synthesis is stimulated in the liver, facilitating the uptake of the yolk within the oocyte and promoting substantial oocyte growth prior to final maturation (Lubzens et al. 2010). The successful completion of this process depends on reproductive strategy, synchrony of oocyte development, spawning frequency, and an adequate presence of essential nutrients (i.e., essential fatty acids and essential amino acids). Essential nutrients are accumulated and transferred to ovaries during vitellogenesis and are responsible for adequate ovocytes development, and later embryology and larval development before exogenous feeding (Bromage et al. 1992). Polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) are crucial for successful reproduction and early life stages development in several fish species (Izquierdo et al. 2001). They represent a source of stored metabolic energy, structural components during organogenesis, and precursors of physiologically active molecules such as prostaglandins and other eicosanoids (Sargent et al. 1995; Tocher 2003). A deficiency of PUFA may affect the patterns of plasma lipids and induce early gonadal atresia, which may reduce the production of gonadal steroids and egg survival (Cerdá et al. 1994). A large pool of free amino acids (FAAs) in the yolk is responsible for reducing the density of the oocytes which help provide buoyancy to the eggs (Rønnestad and Fyhn 1993; Rønnestad et al. 1996; Thorsen et al. 1993).

The previously mentioned research (Mejri et al. 2019, Luck et al. 2019) provided the first comparison of reproductive physiology at non-spawning and PSA sites at one island (Grand Bahamas) and at one spawning month. The goals of this study therefore were to characterize ovarian development and reproductive hormones of bonefish and to quantify the nutritional condition of developing eggs during the spawning season, on a wider geographic scale, including three locations where aggregations are known to occur in the Bahamas. The specific objectives of this study were to (1) compare reproductive maturity and sexual hormone variability between flats and PSA habitats, across geographically distinct locations in the northern and central Bahamas; (2) characterize reproductive phases for female bonefish at flats and PSAs during the spawning season; and (3) characterize the nutrient composition of mature oocytes in each location. This study was conducted at three different locations within the Bahamian Archipelago where bonefish are commonly found: the east end of Grand Bahama, the eastern side of Central Andros, and the eastern side of South Andros. Each of these locations is relatively pristine, contains substantial flats habitat, and has a single identified PSA.

Materials and methods

Sample collection

In the field, oocytes and blood were collected from adult bonefish females from Grand Bahama (March and April 2018), Central Andros (December 2017), and South Andros (January 2018), The Bahamas, during the days immediately surrounding (± 2 days) full moons of known spawning months (Fig. 1). At each island, fish were collected from two general habitat types: (1) shallow tidal flats less than 1 m deep ($n \geq 1$ flat per island), and (2) a PSA located in water greater than 4 m deep ($n = 1$ PSA per island) (Fig. 1). Individuals on the tidal flats were captured using a 50 m × 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. Fish in the PSA were captured via hook and line. Fish were not anesthetized prior to handling in an attempt to reduce handling time and associated stress. Instead, fish were held inverted to achieve a catatonic like state before sampling. Bonefish females were cannulated for oocytes (volume of 1–2 ml) using a soft-tube catheter (Bard 100% latex-free 133 infant feeding tube, 8Fr (2.27 mm diameter, 26 cm length) attached to a 3-ml syringe barrel. Once back in the lab, about two thirds of the oocytes were frozen at − 80 °C for nutrient analysis and one third were preserved in 10% neutral buffered (NB) formalin for histological analysis. In the field, blood was drawn from the ventral side of the fish's caudal vein using a heparinized syringe and deposited into a lithium heparin lined BD vacutainer™. Back in the lab, immediately after the field work, plasma was then separated from blood by centrifugation (2500 rpm for 20 min) and stored at − 80 °C until specific assays could be performed. In total, 49 females (ranging from 373 to 640 mm FL) were sampled from flats ($n = 30$ fish) and PSAs ($n = 19$ fish) during the spawning season (Table 1).

Histological preparation of oocytes

Oocytes stored in 10% NB 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 was used to cut 8–10 μm thick sections from the embedded samples that were mounted on pre-labeled glass slides and stained with hematoxylin and eosin for examination. A subset of oocytes ($n \geq 60$ oocytes) from each female was photographed from each histology sample using an OLYMPUS BX51 microscope at total magnifications between × 40 and × 100.

Reproductive phase classification

For each subset, developmental stages of oocytes were categorized as vitellogenic (Vtg), 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. Secondary development occurs through vitellogenesis and results in Vtg oocytes. Vitellogenic oocytes were identified by their relatively large size, yolk accumulation within the cytoplasm, and the presence of cytoplasmatic inclusions (i.e., oil droplets). The latter were measured for diameters (μm). In total, 9232 oocytes were categorized, and 1907 Vtg

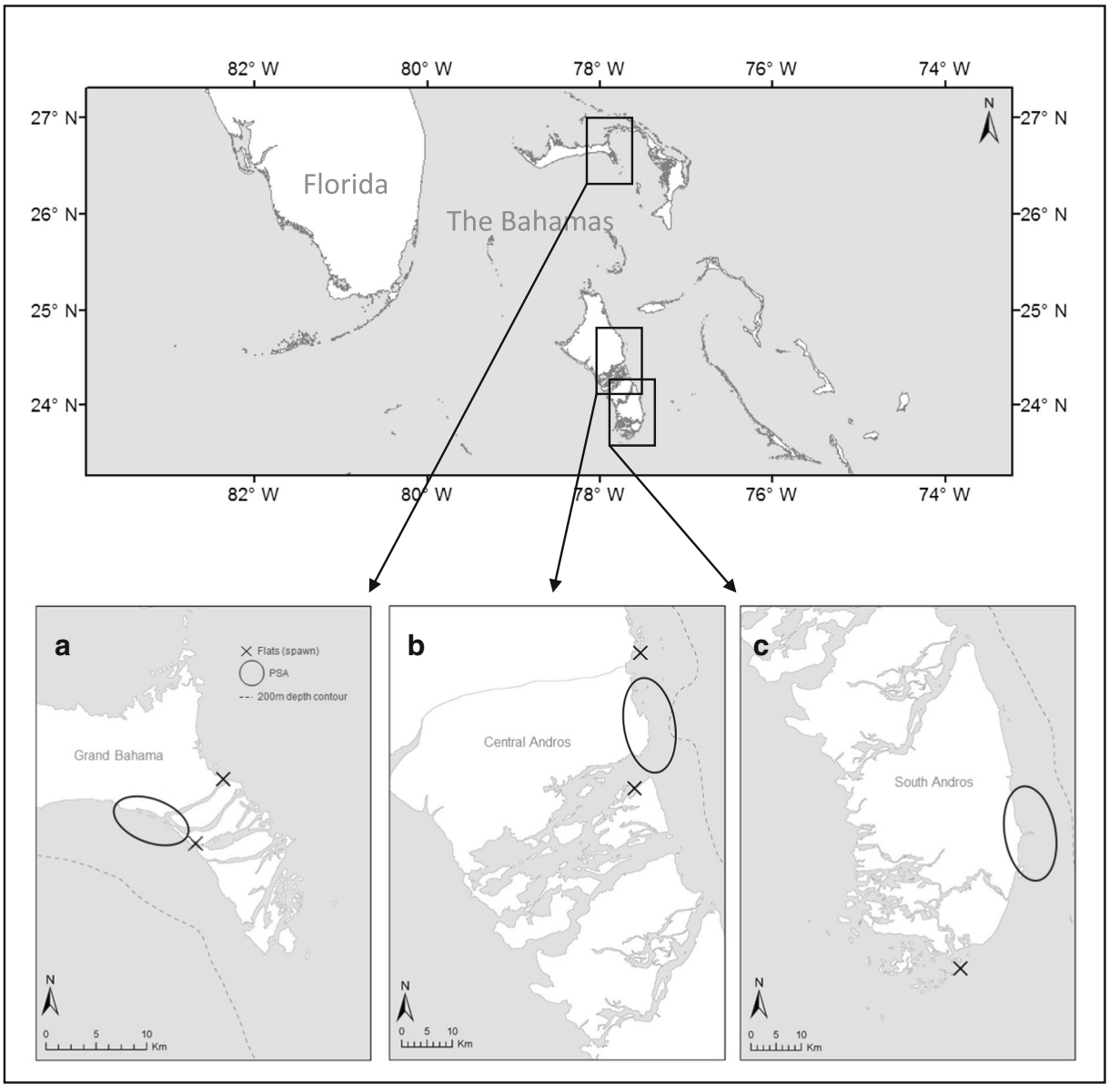

Fig. 1 Map showing sampling locations across three islands in The Bahamas, and the respective flats and pre-spawn aggregation (PSA) habitat sampled within each. Flats habitat was sampled during both spawning and non-spawning months and has been labeled as flats (spawn) and flats (non-spawn) respectively. All islands were sampled during the full moon of a spawning month ([A] Grand Bahama: March and April 2018; [B] Central Andros: January 2018; and [C] South Andros: December 2017). For conservation purposes, the PSA locations are not precisely located, but do occur within the ellipses

oocytes were measured. Moreover, oocytes were evaluated to classify the state of reproductive development at the time of sampling using the method developed by Lowerre-Barbieri et al. (2011). This approach categorizes individuals along a reproductive development gradient based on the relative proportion and the presence/absence of certain oocyte stages. To complete this analysis, Vtg oocytes were further separated into three substages (primary [Vtg1], secondary [Vtg2], and tertiary [Vtg3]) based on oocyte diameter, amount of yolk within cytoplasm, and the presence and appearance of oil droplets (Fig. 2). Fish were then categorized as "immature," "developing," "spawning capable," and "regressing" based on the relative occurrence of each oocytes stage (Brown-Peterson et al. 2011). The categorization of individuals in this way allowed for

Table 1 Sample sizes for all fish blood plasma (hormone) and oocyte (histology and biochemistry) collected at each island (Grand Bahama, Central Andros, and South Andros, The Bahamas), habitat (flats, PSA), and month/year. Please note that the total number for each month and each location does not reflect the total of fish sampled. For example, in March 2018, from Grand Bahama, at the flats, 2 plasma samples and 2 egg samples were collected for hormone analyses and for histology and biochemistry: this means samples were collected from 2 fish in total and not 4

	Grand Bahama				Central Andros		South Andros	
	March 2018		April 2018		December 2017		January 2018	
Habitat	PSA	Flats	PSA	Flats	Flats	PSA	Flats	PSA
Plasma	1	2	6	7	5	6	16	6
Eggs	1	2	6	7	0	0	16	6

histological and hormonal metrics to be coupled for a finer scale evaluation of bonefish reproductive development.

Hormone plasma level determination

17β-estradiol (E2) and testosterone (T) concentrations were quantified via enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical Company, USA). The plasma sample (100 μl) was extracted based on the manufacturer specifications (Cayman Chemical Company, USA). Samples were run at two dilutions to minimize interference within wells. Plates were analyzed via absorbance at a wavelength of 405 nm using a microplate reader (Biotek, Synergy H1, USA). Luteinizing hormone (LH) was quantified via enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource, USA, catalog #: MBS733861). Fifty microliters of sample plasma, 50 μl HRP conjugate, and 50 μl antibody were added to each well. These reagents were mixed and then incubated for 2 h at 37 °C. The optical density of the solution in each well was then determined via absorbance within 5 min using a microplate reader (Biotek, Synergy H1, USA) set to a wavelength of 450 nm.

Biochemical analysis

Oocyte lipids were extracted according to procedures developed by Folch et al. (1957) and modified by Parrish (1999). Lipid extracts were separated into neutral and polar fractions using silica gel column (30 × 5 mm i.d., packed with Kieselgel 60, 70–230 mesh; Merck, Darmstadt, Germany) hydrated with 6% water, and eluted with 10 mL of chloroform:methanol (98:2 v/v) for neutral lipids followed by 20 mL of methanol for polar lipids (Marty et al. 1992). The neutral lipid fraction was further eluted on an activated silica gel with 3 mL of hexane and diethyl ether to eliminate free sterols. All fatty acid methyl esters (FAME) were prepared as described by Lepage and Roy (1984). An internal standard corresponding to 2.5 μg of C19:0 was added to each vial before samples were analyzed using the MIDI Sherlock® Microbial Identification System (MIS). The MIS uses 5890, 6890, or 6850 gas chromatographs (column, ultra 2 column-A 25 m × 0.2 mm phenyl methyl silicone fused silica capillary column; gas chromatograph, T ramps from 170 to 270 °C at 5 °C per 1 min). The Sherlock MIS uses an external calibration standard developed and manufactured by Microbial ID, Inc.

For free amino acid analysis, samples of oocytes were ground and diluted with 2 mL distilled water. A 10-μl (2.5 nmoles) internal standard (two internal standards Norvaline, Sigma # N7502 [for primary amino acids] and Sarcosine, Sigma # S7672 [for secondary amino acids]) were added to all samples, standards, controls, and blanks at the beginning of the assay. Standards, controls, and samples (30 or 15 μl of sample) were added to all injection vials and mixed well. Amino acids were derivatized and separated on an Agilent 1260 liquid chromatograph (LC) with "Chemstation" software that controls the LC and collects, analyzes, and reports the data. In this assay, cysteine is not quantitated. Tryptophan was obscured by the very large doubly derivatized Taurine. Amino acid analyses were provided by Protein Chemistry Laboratory (Texas A&M University, TX).

Statistical analysis

Mean diameter of vitellogenic oocytes and 17β-estradiol, testosterone, and LH concentrations were compared across both island (Grand Bahama, South Andros, and Central Andros) and habitat (Flat and PSA) using a two-way

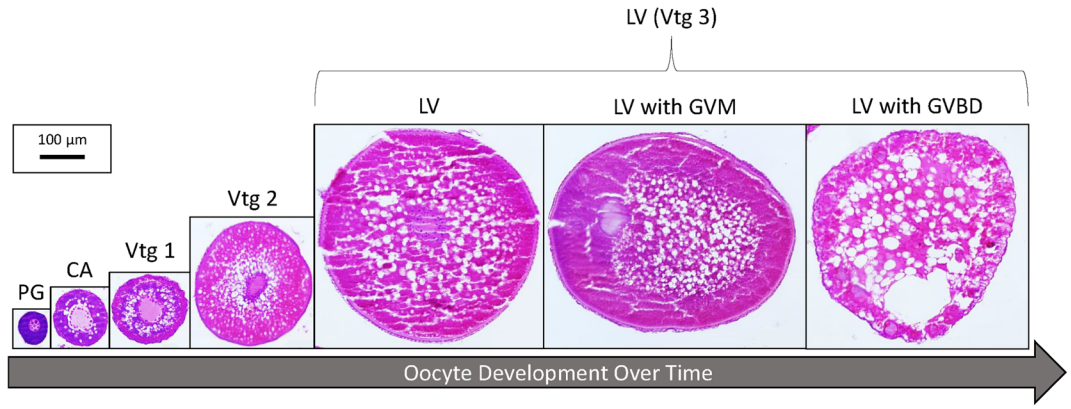

Fig. 2 Chronological schematic of oocyte stages present during the reproductive development of bonefish (*Albula* spp.) including primary growth (PG), cortical alveolar (CA), primary vitellogenic (Vtg 1), secondary vitellogenic (Vtg 2), tertiary vitellogenic (LV), LV with germinal vesical migration (GVM), and LV with germinal vesicle breakdown (GVBD). This classification is based on the classification scheme developed by Crabtree et al. (1997)

nested ANOVA. Data were log-transformed to meet parametric assumptions of normality and homoscedasticity, when necessary. Both factors ("habitat" and "island") were treated as fixed factors with "habitat" nested within "island." The significance threshold for all analyses was set at $P < 0.05$.

17β-estradiol and testosterone concentrations were compared among reproductive phases using a one-way ANOVA following log transformation of hormone data. Analysis only included individuals sampled along flats habitat of all three islands sampled. All ANOVA tests were performed using R software (R Core Team 2014).

The frequency of occurrence of the three predominant oocyte stages (PG, CA, and Vtg), fatty acid (FA) profiles from neutral and polar lipids, and FAA profiles were compared across islands (Grand Bahama, Central Andros, and South Andros) and habitats (Flats and PSA) using a nested 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 (arcsine square root) when necessary.

Results

Histology

Mean vitellogenic oocyte diameter did not significantly differ across all islands (nested two-way ANOVA; $F_{(2, 31)} = 1.21$, $P = 0.31$). However, mean vitellogenic oocyte diameter was significantly larger (nested two-way ANOVA; $F_{(3, 31)} = 8.70$, $P = <0.001$) in fish sampled at the PSA (480 ± 67 μm) compared to those sampled along the flats (308 ± 35; Fig. 3).

Nested PERMANOVAs indicated that frequencies of oocyte type differed significantly by both island (pseudo-$F_{(2, 44)} = 3.99$, $P < 0.05$) and habitat (pseudo-$F_{(3,44)} = 5.38$, $P < 0.001$). Differences between islands were driven by a significantly higher proportional occurrence of PG oocytes from females in South Andros compared to those from Grand Bahama ($P = 0.01$; Fig. 4). Differences between flats and PSAs were driven by both a higher occurrence of PG in fish sampled along flats compared to those at the PSA ($F_{(3, 44)} = 5.13$, $P < 0.01$) and an inversely higher occurrence of vitellogenic oocytes in fish at PSAs compared to those from flats ($F_{(3, 44)} = 7.20$, $P < 0.001$; Fig. 4).

Sex hormone concentrations

Across all islands, significant differences in mean concentration of 17ß-estradiol were observed (one-way ANOVA; $F_{(2, 43)} = 4.13$, $P < 0.05$). Comparison between flats and PSAs within islands revealed that mean concentrations of 17ß-estradiol were significantly higher at the PSA compared to flats habitat ($F_{(3, 43)} = 15.29$, $P < 0.001$) (Fig. 5). Testosterone levels were not significantly different when compared across all islands ($F_{(2, 37)} = 1.60$, $P = 0.215$). Within each island, testosterone concentrations were significantly higher in fish sampled from the PSA compared to those sampled

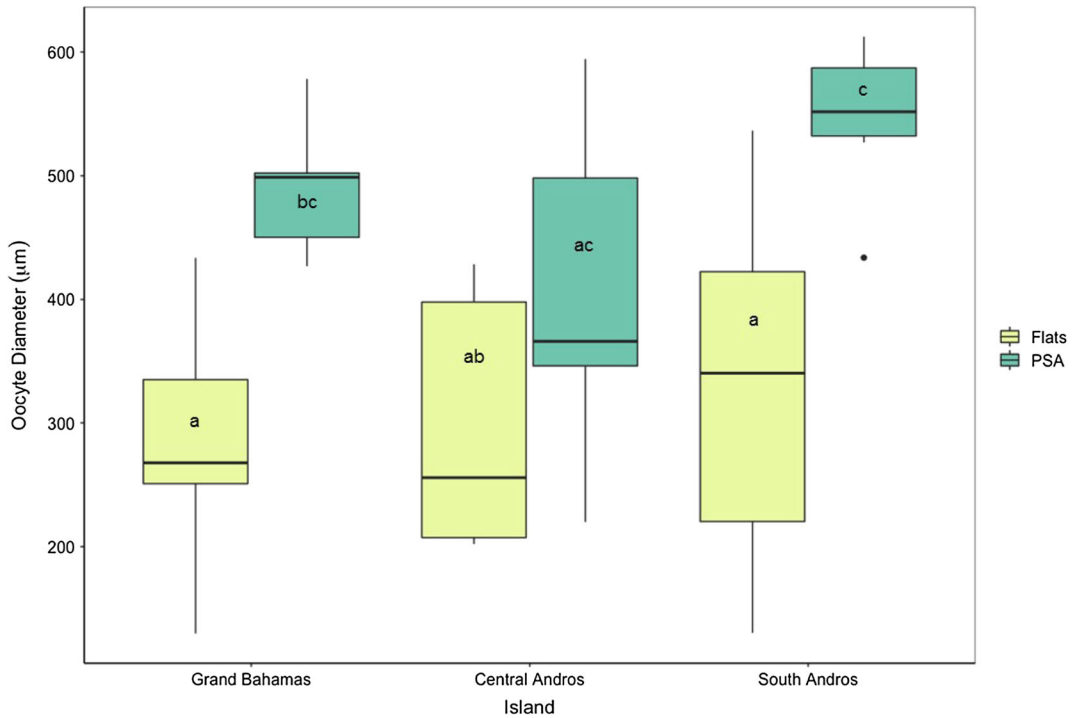

Fig. 3 Diameters of vitellogenic oocytes collected from female bonefish (Albula spp.) sampled at two different habitats (flats and pre-spawning aggregation [PSA]) across three different islands (Grand Bahama, Central Andros, and South Andros, The Bahamas). The top and bottom borders of boxes indicate upper and lower interquartile range of data for each habitat type sampled. The horizontal black line within box indicates median oocyte diameter for each habitat type sampled. Vertical lines indicate maximum and minimum oocyte diameters observed for each habitat type sampled. Letters indicate significant differences in mean oocyte diameters between habitat types across island (two-way ANOVA)

along flats ($F_{(3,\ 37)} = 20.75$, $P < 0.001$; Fig. 5). LH levels did not significantly differ when compared across all islands ($F_{(2,\ 24)} = 2.36$, $P = 0.115$). However, within each island, LH levels were significantly higher at PSAs compared to flats habitat ($F_{(3,\ 24)} = 3.43$, $P = 0.032$; Fig. 5).

All fish sampled at PSAs were spawning capable with 88.6% of spawning capable fish showing evidence of germinal vesicle migration (GVM) or breakdown (GVBD). Conversely, 29.0% of fish sampled along the flats were spawning capable with only 3.1% exhibiting evidence of GVM or GVBD.

Since mean 17β-estradiol and testosterone concentrations found in bonefish sampled along flats did not significantly differ by island (one-way ANOVA: $F_{(2,\ 27)} = 0.92$, $P = 0.41$), samples collected from the flats were binned based on a histologically determined reproductive phase and hormone profiles compared. Significant differences in mean 17β-estradiol were found among reproductive phases exhibited by bonefish sampled along flats ($F_{(3,\ 25)} = 5.90$, $P < 0.01$). This difference was driven by significantly lower 17β-estradiol levels in "immature" and "regressing" fish compared to those "developing" or "spawning capable" ones (Fig. 6). Mean testosterone concentration of Bonefish sampled along flats did not significantly differ by reproductive phase ($F_{(3,\ 24)} = 2.53$, $P = 0.08$; Fig. 6).

Fatty acid composition

Due to unforeseen logistical constraints, we were not able to collect enough oocyte samples for nutrient analysis from the Central Andros location. Neutral and polar fatty acid lipids from bonefish oocytes had similar composition when compared within islands and habitats: neutral FA lipids: pseudo-$F_{\text{Island}\ (1,\ 38)} = 5.35$, $P = 0.34$; pseudo-$F_{\text{Habitat}\ (2,\ 38)} = 0.85$, $P = 0.51$ and polar FAs lipids: pseudo-$F_{\text{Island}\ (1,\ 38)} = 2.94$, $P = 0.33$; pseudo-$F_{\text{Habitat}\ (2,\ 38)} = 1.46$, $P = 0.19$. Overall, within neutral lipids, monounsaturated fatty acids (MUFAs) made up the larger fraction (> 42% of total neutral FA) compared to

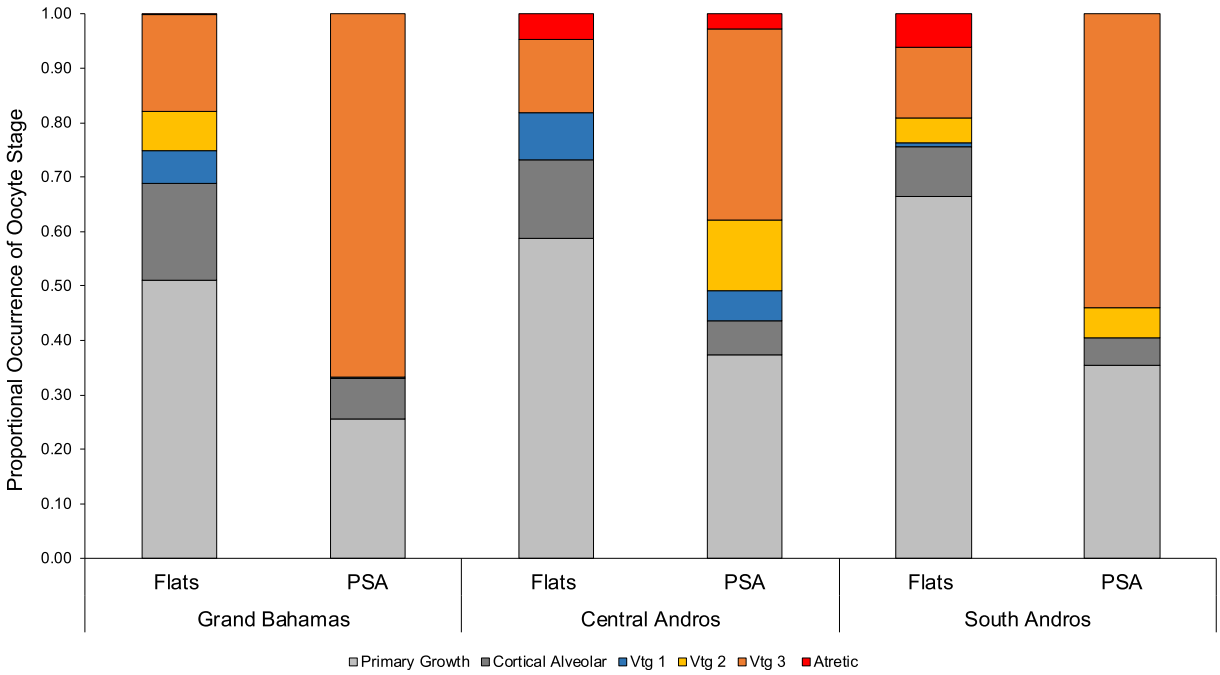

Fig. 4 Proportional distribution of primary growth (PG), cortical alveolar (CA), primary vitellogenic (Vtg 1), secondary vitellogenic (Vtg 2), tertiary vitellogenic (Vtg 3), and atretic oocytes found in female bonefish (*Albula* spp.) sampled from both the flats and PSAs of Grand Bahama, Central Andros, and South Andros, The Bahamas

saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) (> 31% and > 20% of total neutral FA, respectively; Table 2). Contrastingly, SFA and PUFA made up the larger fraction in polar lipids (> 36 and > 43% of total polar FA, respectively; Table 2). The fatty acid profiles of bonefish oocytes were characterized by high levels of palmitic FA (16:0) and oleic FA (18:1 n-9) in both lipid fractions (neutral and polar) (Table 2). However, the three essential FA, ARA, DHA, and EPA, were higher in the lipid polar fraction. DHA was the highest with relative percentages as high as 23% of total polar FA, ARA and EPA were > 13 and > 4% of total polar FA, respectively; Table 2).

Free amino acid composition

Eight essential free amino acids (FAA); histidine (HIS), isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET), phenylalanine (PHE), threonine (THR), and valine (VAL) were identified in bonefish oocytes (Table 3). FAA profiles varied significantly between islands (pseudo-$F_{\text{Island (1, 55)}} = 17.02$, $P = 0.001$) but did not vary between habitat type within each island (pseudo-$F_{\text{Habitat (2, 55)}} = 1.67$, $P = 0.20$). SIMPER analysis showed that HIS and taurine (TAUR) explained up to 47% of the differences between Grand Bahama and South Andros islands. TAUR and HIS levels were seven and two times higher, respectively, in oocytes collected

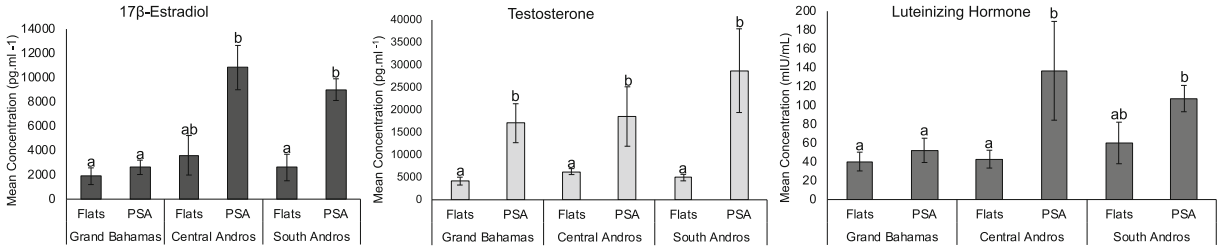

Fig. 5 Spatial variation in 17β-estradiol, testosterone, and luteinizing hormone concentrations (mean ± SEM) for bonefish (*Albula* spp.) sampled across three islands (Grand Bahama, Central Andros, and South Andros, The Bahamas) and two habitat types (flats and PSA) within each island. Black letters indicate significant differences (two-way ANOVA)

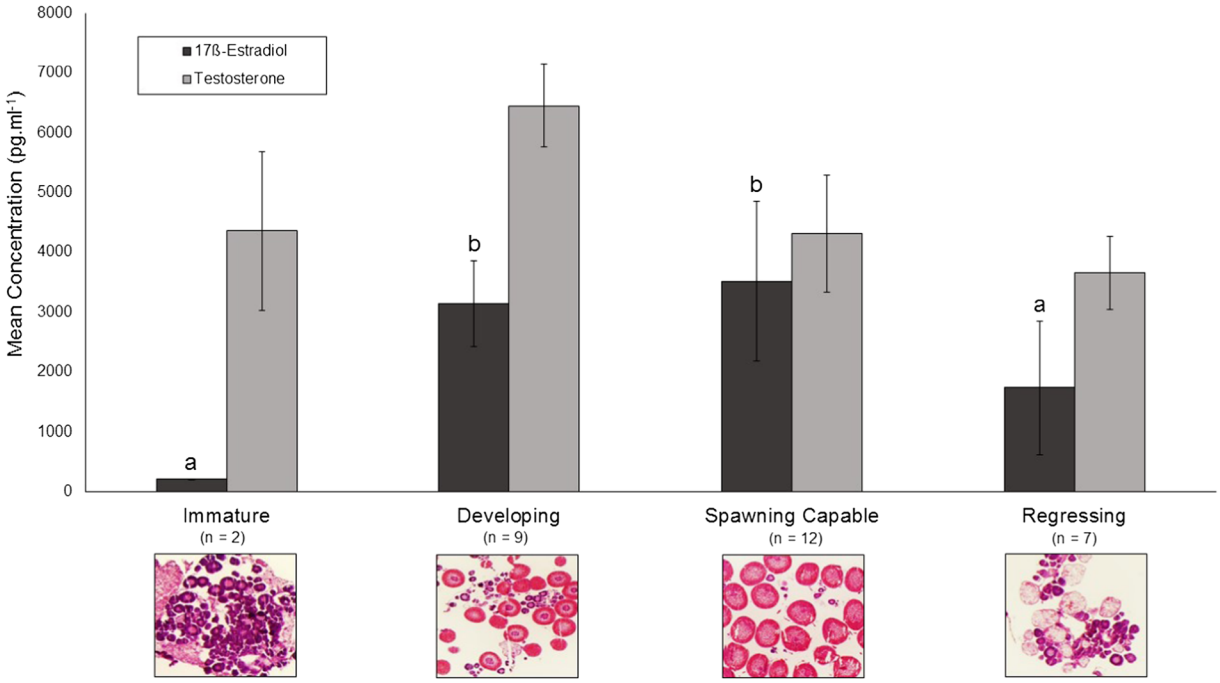

Fig. 6 Concentrations of 17β-estradiol and testosterone (mean ± SEM) for each reproductive phase observed in female bonefish (*Albula* spp.) sampled on flats across all islands (Grand Bahama, South Andros, and Central Andros, The Bahamas). Letters indicate significant differences in mean concentration of 17β-estradiol across phases (one-way ANOVA). No significant differences were observed for testosterone

from Grand Bahama compared to those from South Andros.

Discussion

The multi-island assessment of female bonefish reproductive development provided a unique opportunity to evaluate the variability in spawning readiness within and across habitat types. The findings of this study not only support the importance of habitat type for reproductively developing female bonefish, but also indicate the potential for variability in this spatial relationship across other islands where bonefish thrive and actively spawn. These results provide valuable insight into the reproductive physiology and ecology of bonefish adults, and the production and quality of eggs that could affect the quality and viability of their leptocephalus larvae.

Reproductive development and hormone levels

Previous findings from Grand Bahama suggested that, based on levels of 17β-estradiol in bonefish, vitellogenesis begins on the flats and continues to occur at the PSA (Mejri et al. 2019; Luck et al. 2019). The present study not only supports these findings, but suggests that the occurrence of vitellogenesis prior to aggregation is a common physiological characteristic of bonefish given the much larger geographic scale of this study. The significantly higher levels of both 17β-estradiol and testosterone found at the South and Central Andros PSAs may have been a consequence of the greater distance between the flats and PSA (~ 30 km) habitat compared to the lower levels found on Grand Bahama island (~ 12 km). Assuming the majority of vitellogenesis is occurring at the flats and during migration to PSAs, it is conceivable that fish are more likely to have significantly different levels of both 17β-estradiol and testosterone between their start (flats) and end (PSA) locations given the greater distance, and therefore, more time available for hormone synthesis to occur. Females may leave flats at the same time, undergoing the same timeline of oocyte development, and show up at the PSA earlier than fish making the same journey from flats further away.

Another possible explanation is based on the relative occurrence of GVM/GVBD oocyte stages at the PSAs. Bonefish females sampled at PSAs in Grand Bahama

Table 2 Neutral and polar fatty acid composition (mean ± SD, expressed as percentage of total neutral and polar lipids detected) in bonefish (*Albula* spp.) oocytes sampled across two islands (Grand Bahama and South Andros, The Bahamas) and two habitat types (flats and PSA) within each island

Fatty acid	Grand Bahama		South Andros	
	Neutral	Polar	Neutral	Polar
C14:0	3.47 ± 0.33	0.74 ± 0.07	3.13 ± 0.41	0.78 ± 0.05
C15:0	1.33 ± 0.04	0.65 ± 0.05	2.39 ± 0.80	0.80 ± 0.02
C16:0	16.57 ± 0.84	22.50 ± 0.74	15.75 ± 1.57	22.87 ± 2.12
C17:0	2.05 ± 0.26	1.71 ± 0.09	2.63 ± 0.26	2.14 ± 0.06
C18:0	7.52 ± 0.36	10.36 ± 0.69	10.88 ± 0.81	11.78 ± 0.74
C16:1	11.50 ± 0.57	3.30 ± 0.56	9.10 ± 0.92	3.16 ± 0.36
C17:1	1.74 ± 0.00	0.69 ± 0.05	1.91 ± 0.25	0.86 ± 0.09
C18:1 n-9	31.06 ± 0.49	10.83 ± 1.00	28.31 ± 0.47	11.75 ± 0.45
C20:1 n-9	2.17 ± 0.84	0.42 ± 0.16	2.27 ± 0.53	0.58 ± 0.57
C18:2 n-6	2.73 ± 0.11	0.81 ± 0.23	2.17 ± 0.31	0.87 ± 0.02
C18:3 n-6	0.39 ± 0.03	0.18 ± 0.00	0.35 ± 0.03	0.21 ± 0.00
C20:3 n-6	0.84 ± 0.14	0.95 ± 0.38	0.82 ± 0.07	1.06 ± 0.01
C20:4 n-6	1.76 ± 0.53	15.30 ± 1.35	4.97 ± 1.84	13.25 ± 2.24
C20:5 n-3	4.67 ± 0.08	4.86 ± 0.22	2.37 ± 0.44	4.28 ± 0.18
C22:6 n-3	7.52 ± 0.32	23.00 ± 0.72	1.38 ± 0.10	16.57 ± 2.35
TOTAL SFA$^{\alpha}$	31.71 ± 0.06	36.39 ± 0.08	35.78 ± 0.91	39.83 ± 0.43
TOTAL MUFA$^{\beta}$	46.59 ± 0.79	16.00 ± 1.68	42.24 ± 0.21	16.35 ± 0.14
TOTAL PUFA$^{\delta}$	21.54 ± 0.90	47.59 ± 1.63	20.69 ± 0.49	43.66 ± 0.38

$^{\alpha}$ Includes 12:0, 13:0, 20:0, and 22:0 whose combined percentages are ≤ 0.2% of total neutral fatty acid lipids

$^{\beta}$ Includes 24:1 n-9, whose combined percentages are ≤ 0.2% of total neutral fatty acid lipids

18:1 n-7, 18:1 n-5, 20:1 n-11, whose combined percentages are ≤ 0.2% of total polar fatty acid lipids

$^{\delta}$ Includes 20:2, 18:3 n-3, and 20:3 n-3 whose combined percentages are ≤ 1% of total neutral fatty acid lipids

18:4 n-3, 19:4 n-6, 20:4 n-3, 22:4 n-6, 22:5 n-6, 22:5 n-3 whose combined percentages are ≤ 0.5% of total polar fatty acid lipids

SFA, saturated fatty acids; *MUFA*, monounsaturated fatty acids; *PUFA*, polyunsaturated fatty acids

showed a lower proportion of spawning capable fish with GVM percentages (~ 66%) lower than South and Central Andros (~ 100%). In other words, while all fish at PSAs were spawning capable, all South and Central Andros spawning capable females sampled had reached the final oocyte maturation stages compared to Grand Bahama. These contrasting levels of oocyte development between islands were likely driven by higher overall concentrations of LH [the primary gonadotropin that drives final oocyte maturation in fish (Nagahama and Yamashita 2008) in fish sampled at the PSAs of both South and Central Andros islands compared to Grand Bahama. Thus, we can conclude that bonefish females at South and Central Andros have begun final oocyte maturation. While it has become generally accepted that bonefish spawn with lunar periodicity (full and new moon; Danylchuk et al. 2011), peak development at the PSA just prior to offshore migration may vary by island. All three islands were sampled through the full moon of a known spawning month, assuming spawning events occur primarily during this time (Adams et al. 2019a, b; Danylchuk et al. 2011). However, the lower mean 17β-estradiol and testosterone values from Grand Bahama PSAs sampled in 2017 (Luck et al. 2019) and 2018 (the current study) combined with the lower levels of LH compared to Central and South Andros indicate peak lunar development at the PSA habitat in Grand Bahama may occur either earlier or later than other islands (before or after full moon, instead of during the full moon). The ability to further address these hypotheses falls outside the scope of this study and would require monitoring of fish movement both to and from

Table 3 Free amino acid composition (percent of total free amino acids; mean ± SD) in bonefish (*Albula* spp.) oocytes sampled across two islands (Grand Bahama and South Andros, The Bahamas)

	Grand Bahama	South Andros
Essential amino acids		
Histidine (HIS)	11.68 ± 3.91	5.25 ± 2.41
Isoleucine (ILE)	2.88 ± 0.76	4.37 ± 0.69
Leucine (LEU)	4.60 ± 1.21	7.09 ± 0.76
Lysine (LYS)	3.43 ± 1.51	5.60 ± 1.04
Methionine (MET)	1.25 ± 0.35	1.86 ± 0.58
Phenylalanine (PHE)	1.74 ± 0.61	2.66 ± 0.48
Threonine (THR)	3.07 ± 1.17	5.07 ± 0.49
Valine (VAL)	3.94 ± 1.17	6.08 ± 1.00
Conditionally essential amino acids		
Arginine (ARG)	3.34 ± 0.94	4.44 ± 0.48
Proline (PRO)	2.63 ± 1.05	4.61 ± 0.60
Tyrosine (TYR)	1.45 ± 0.63	2.56 ± 0.33
Non-essential amino acids		
Alanine (ALA)	6.96 ± 2.11	12.00 ± 1.08
ASX*	4.02 ± 1.90	7.44 ± 0.76
GLX*	9.60 ± 1.76	9.49 ± 1.86
Glycine (GLY)	4.35 ± 2.17	8.11 ± 1.12
Serine (SER)	6.40 ± 1.60	8.96 ± 1.82
Taurine (TAUR)	28.46 ± 12.30	4.41 ± 2.91

ASX*, includes aspartic acid (ASP) and asparagine (ASN)

GLX*, includes glutamine (GLN) and glutamic acid (GLU)

PSAs as well as offshore movement at several islands in concert with repeated hormonal and histological sampling. Such an approach could reveal a more specific timeline of what is occurring physiologically in bonefish during late stage development.

Within flats habitat, substantial variability was observed in the reproductive state of females during the spawning season at all three islands. The occurrence of four major reproductive phases (immature, developing, spawning capable, and regressing) commonly observed in reproductively active group synchronous marine spawners (Ganias and Lowerre-Barbieri 2018; Lowerre-Barbieri et al. 2011) suggests that flats not only provide normal home range habitat year-round (Boucek et al. 2019), but also expose individuals to the abiotic variables (temperature, salinity, photoperiod, etc.) that influence oocyte development (Hansen et al. 2001; Holt et al. 2007; Mazzeo et al. 2014). The use of non-spawning habitat for initial oocyte development prior to spawning related movement has been observed in many species of batch spawning marine fishes including spotted seatrout (*Cynoscion nebulosus*), and tarpon (*Megalops atlanticus*) (Crabtree et al. 1997; Lowerre-Barbieri et al. 2011). Theoretically, this strategy allows energetically expensive processes such as vitellogenesis to occur where food is readily available and predatory threats are minimal prior to moving towards spawning habitat. The technique used for nutrient composition and profiling for these bonefish's oocytes was instrumental in confirm this partitioning behavior.

Nutrients

Analysis of the lipid and free amino acid composition of oocytes enables characterization of the energy sources available to embryos and the metabolic precursors for embryonic and early larval development. The relative abundance of FA neutral lipid fraction (% of total neutral FA content) in bonefish oocytes was dominated by MUFA (44.5%) followed by SFA (34%), with oleic acid (18:1 n-9) and palmitic acid (16:0) compromising up to 31% and 17% of these fractions, respectively. Oleic acid is generally used as an energy source during early development from fertilized eggs to yolk-sac larvae in species such as Senegalese sole (*S. senegalensis*) and dentex porgies (Sparidae: *Dentex dentex*) (Samaee et al. 2009; Vázquez et al. 1994). The MUFA and SFA contained within eggs must also provide sufficient energy reserves for initial larval swimming and prey capture as they transition to exogenous sources of nutrition. Polar lipids provide structural components for developing embryos. In the polar lipid fraction, PUFAs were the most abundant (45.6%) followed by SFA (38%) and MUFA (16.4%), and were approximately in agreement with previous findings on this species (Mejri et al. 2019). SFA and PUFA are important components of cell membrane lipids and in the build-up of oocytes during vitellogenesis (McKenzie et al. 1998; Sargent et al. 2002). The higher relative abundance of ARA than EPA in bonefish oocytes (Table 2) was similar to a previous study carried out on this species at different flat locations in Grand Bahama, The Bahamas (Mejri et al. 2019) and with findings of Yanes-Roca et al. (2009) on common snook (*Centropomus undecimalis*). Previous studies carried out on eggs of Japanese eel (*Anguilla japonica*) found a positive relationship between the relative abundance of ARA and survival rate, blastomere symmetry, and hatch rate (Furuita et al.

2003). This is not surprising since ARA is the major eicosanoid precursor in fish cells that is important in the control of ovulation, embryogenesis, development of the immune system, hatching, and early larval performance (Sargent et al. 2002). DHA was the most abundant of the essential fatty acids, and other studies have shown that DHA has a major role in the formation and structure of membranes in the brain and retina (Wiegand 1996). Studies of the morphological development of the leptocephalus larvae of European eel (*Anguilla anguilla*), which share the same larval type with bonefish, have shown that the retina of small larvae dominated the volume of eyes (Pedersen 2003; Rønquist Knutsen 2015), concluding that vision, among other senses, appeared to be well developed to allow the larvae to actively search for their preferred food or to avoid predators. Thus, a positive relationship between the high abundance of DHA in bonefish eggs may serve to facilitate visual development for leptocephalus larvae.

Our data showed that although FAA profiles varied within islands, they had a similar general profile for several essential and non-essential FAA and are dominated by neutral amino acids such as leucine, valine, isoleucine, alanine, and serine. These findings are in concordance with earlier observations of eggs for 23 species of marine tropical fishes (Rønnestad et al. 1996). One interesting observation in this study was the remarkably higher proportions of HIS and TAUR in oocytes collected from Grand Bahama, compared to those from South Andros. Histidine is an essential amino acid for fish and plays important roles in homeostasis maintenance and osmoregulation (Li et al. 2009; Nagasawa et al. 2001; Rhodes et al. 2010; Sarih et al. 2019) and is particularly important for reproductive success as it is an abundant amino acid in gonads during spawning of certain species (Qari et al. 2013). Moreover, HIS is preferentially retained over other amino acids during early larval development, suggesting the importance of adequate levels in fish oocytes. Additionally, taurine is known to be an important osmoeffector, which participates in the hydration of pelagic eggs before ovulation (El-Sayed 2014). It contributes to enhance lipid metabolism, larval morphology, development, growth, and survival of marine fish larvae in addition to its important role in retinal development and visual system (El-Sayed 2014). Thus, the higher percentages of HIS and TAUR in oocytes from Grand Bahamas suggest that females from that island might have enhanced spawning quality, with possible improved development and survival for embryos and larvae. However, this hypothesis requires further investigation via experimental approaches to elucidate the role of these FAAs in bonefish early life history.

The nutrient requirements of leptocephalus larvae are considered one of the largest mysteries in relation to their ontogeny. It has previously been observed that yolk-sac larvae of European eel have an especially high level of lipase and aminopeptidase enzymes, even from the very early stages (Mazurais et al. 2013), levels that are much higher than what is found in other pelagic fish larvae, which is an indication of the great importance of lipids and amino acids for the earliest life stages of these species. Our findings of rich compositions of FA and FAA in bonefish oocytes suggest that the larvae may share this characteristic with the European eel leptocephalus.

Conclusion

The findings from this study not only expand upon our limited understanding of the seasonal reproductive dynamics of aggregate spawning marine fishes, but also provide a unique viewpoint on the physiological differences between two bonefish habitats during the spawning season. In this study, we assessed hormonal, developmental, and nutrient composition variability across three geographically separate areas where bonefish occur and where PSAs have been identified. Our results indicate that at each island sampled, PSAs play a critical role in facilitating the final stages of vitellogenesis and the onset of final maturation. The differences in testosterone, 17β-estradiol, and LH between Grand Bahama and Andros PSAs suggest variability in peak spawning times may be occurring and require significant further investigation into understanding aggregation periodicity. The results of this study also highlight the importance of flats as critical habitat for reproductive development based on the wide range of progressive development observed in reproductively active females. Additionally, given the known importance of DHA, TAUR, HIS, and ARA in ovulatory and embryonic developmental processes, the findings of this study provide tremendous insight into the requirements of FAA and FA in the ontogeny of bonefish.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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