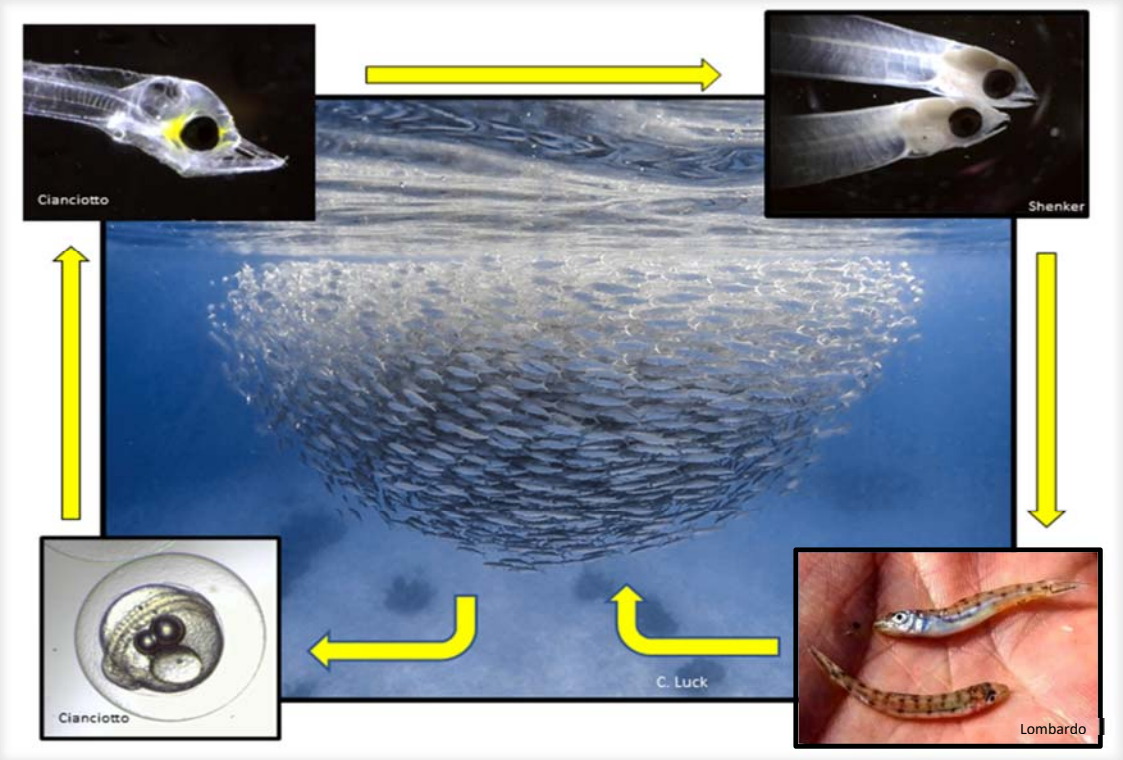
BONEFISH REPRODUCTION RESEARCH PROJECT

MARCH 2016- FEBRUARY 2022

FINAL REPORT



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Acknowledgements

The Bonefish Reproduction Research Program (BRRP) was a very ambitious six-year undertaking that required tremendous support and dedication from a very large and diverse group of organizations and people.

The Bonefish and Tarpon Trust (BTT) and the National Fish and Wildlife Foundation (NFWF) provided the primary funding for this research. We are very appreciative of their support and flexibility that allowed us to adapt efforts in response to research results. BTT also provided support for many specific endeavors throughout the project, with help ranging from collecting broodstock to reaching out to the BTT membership that is fascinated with all things bonefish.

Florida Atlantic University-Harbor Branch Oceanographic Institute (FAU-HBOI) provided the infrastructure research base for all phases of the adult and larval aquaculture studies, as well as physiological, biochemical and genetic analyses of field and laboratory specimens. A group of highly dedicated and hard-working BRRP and facility support staff at FAU-HBOI helped maintain the fish, culture systems and laboratories. Administrative staff coordinated the logistics and funding for national and international travel and ensured that our activities met national animal care rules and regulations. The Media Relations Department did a spectacular job distributing news of our results through a complex web of media to millions of people.

As our research results indicated an increasing need to determine reproductive behavior and physiology of wild fish, a variety of organizations provided us with access to Prespawning Aggregation sites. The East End Lodge on Grand Bahama Island and Bairs Lodge on Andros Island hosted our earliest field studies to determine if bonefish could be induced to spawn in captivity. Subsequent work at the Cape Eleuthera Institute on Eleuthera Island provided excellent culture facilities and a great staff that enabled us to succeed with the first induced spawn of bonefish, as well as the first examination of embryo and early larval development. Support from BTT and NFWF, along with the success in these early endeavors, led the Fisheries Research Foundation to award us with ship time amounting to seven research cruises aboard their M/Y *Albula* to study bonefish spawning biology and behavior at spawning sites near many islands in the Bahamas. Captain Tom Henshilwood and his crew provided us with the ability to take the laboratory to the fish, where we set up adult spawning and larval culture tanks right next to a spawning aggregation, and where we could use small boats to track acoustically tagged fish all night as they went on long distance and deep spawning migrations. Marine biologists usually don't even dare to dream of having access to such a wonderful ship and crew.

Sea World, Orlando, and the Florida Keys Aquarium Encounter, in Marathon FL, graciously agreed to adopt the bonefish broodstock from HBOI at the end of the project, and to transport them to their new homes where they will be able to enthrall a new generation of fish enthusiasts.

Executive Summary

The Bonefish Reproduction Research Project (BRRP) was initiated in March 2016 as a joint project between Bonefish and Tarpon Trust (BTT) and National Fish and Wildlife Foundation (NFWF). This project focused on creating the ability to help reverse the decline of the very valuable recreational bonefish fishery in south Florida and the Florida Keys, and to provide information to contribute to fishery and habitat management strategies to protect spawning sites and populations in Florida and around the Bahamas and the Caribbean.

Throughout the project we followed two parallel yet highly inter-linked tracks: 1) the development of aquaculture techniques at Florida Atlantic University's Harbor Branch Oceanographic Institute (FAU-HBOI) to determine how to maintain bonefish in captivity, induce spawning, and produce and study eggs, larval, and juvenile bonefish development; and 2) the study of the reproductive physiology and biology of wild populations of bonefish, including natural spawning behavior, larval production and transport, and identification of juvenile nursery habitats. Results overall have exceeded expectations, but as with any scientific endeavor new questions have arisen due to the successes and difficulties encountered along the way.

Funding from BTT and NFWF led to the successful leverage of a grant from the Fisheries Research Foundation for ship time aboard the M/Y *Albula* to study bonefish spawning in the Bahamas during the 2018-19 and 2019-20 winter spawning seasons. The ship provided excellent sustained access to PSA sites, with its tending vessels and crew facilitating adult fish collection and extended on-water operations with its technological capabilities (e.g., side-scan sonar). Additionally, on-board culture facilities were established for studies on hormonal induction of the final stages of egg maturation, spawning, and embryo and early larval development. This work

complemented our shore-based studies on reproductive physiology of wild bonefish populations at several Bahamian spawning sites and provided a framework to inform the controlled spawning

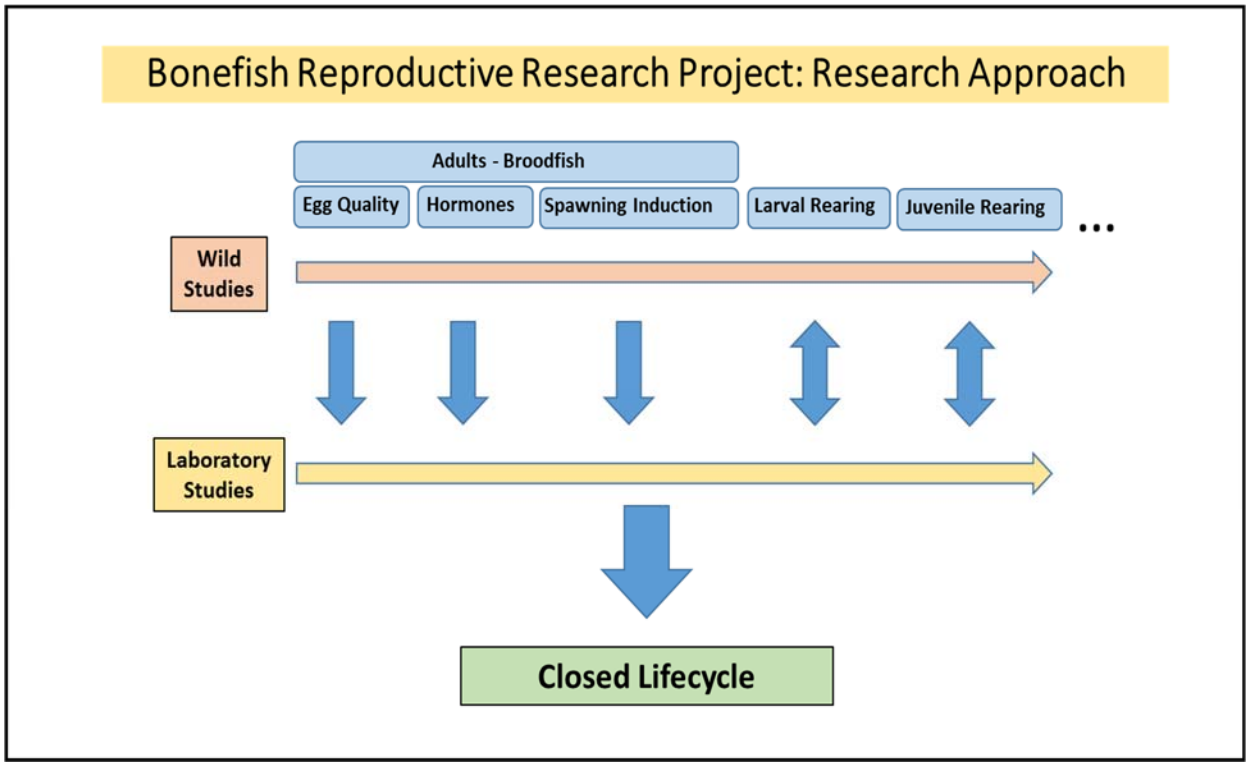


Figure 1. Graphical conceptualization of the parallel wild and laboratory research approach applied to accelerate information acquisition for the BRRP (arrows represent information flow).

methods (Figure 1) for bonefish broodstock at the aquaculture facility at FAU-HBOI.

Prior to the BRRP studies, much of the biological information that is necessary to understand spawning, larval husbandry and life history, and juvenile husbandry were unknown. The BRRP has substantially filled in these knowledge gaps and has made a quantum leap in advancing the understanding of bonefish biology (Figure 2) particularly in the realm of adult reproductive and juvenile biology.

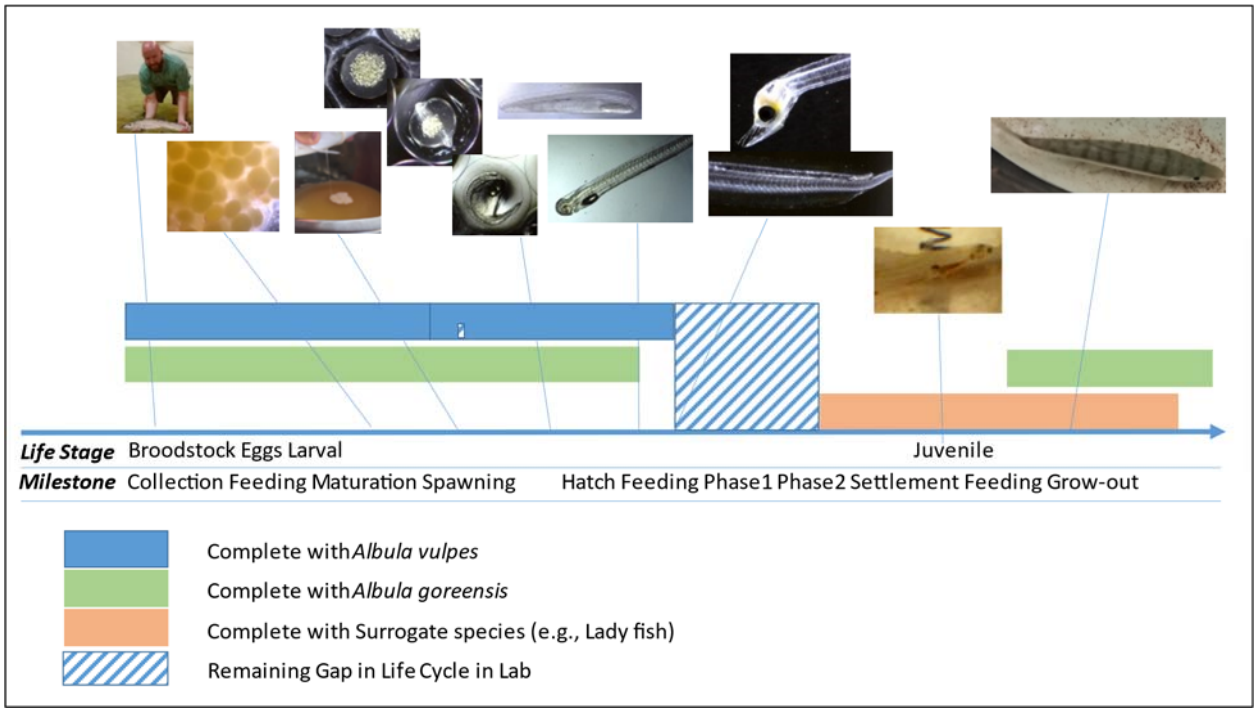


Figure 2. Knowledge gaps in the understanding of bonefish life history filled by the BTT BRRP project.

Highlights of the BRRP achievements throughout the project include:

1) Met the challenge of creating husbandry protocols that allowed keeping captive bonefish in tank conditions suitable for research of spawning induction
    a. Maintained multiple groups of *Albula vulpes* and *Albula goreensis* broodstock over the course of the project at HBOI for experiments to achieve environmentally and hormonally induced spawning.
    b. Developed methods for shortened "annual" cycles in temperature and photoperiod to allow the availability of reproductively ready fish for experimentation throughout the year rather than seasonally.
2) Documented physiological characteristics and environmental requirements of wild bonefish that explained the reproductive process

a. Determined reproductive hormone levels and developing egg nutrient composition of wild bonefish from the Bahamas, producing data that were used to inform the laboratory studies at HBOI.

b. Determined through seasonal and spatial changes in hormone levels that vitellogenesis (finalization of egg maturation before ovulation and spawning) still occur when fish are transitioning from Pre-Spawning Aggregation (PSA) to spawning.

c. Demonstrated that bonefish follow group-synchronous ovarian development and produce lipid-rich eggs, necessary to fuel energetic demand for embryogenesis, early larvae development, and buoyancy.

d. Demonstrated that PSA locations, across geographically separate areas, through hormonal, developmental, and nutrient composition assessments, play a critical role in facilitating the final stage of vitellogenesis and the onset of final maturation.

e. Characterized movements of pre-spawning fish and tracked them throughout a spawning run from the PSA to their deep-water spawning site using active acoustic telemetry. Through tracking of tagged fish, and periodic observation of the school on sonar, fish in the PSA were found to move at night off the edge of the reef, where they dove as deep as 137 m and remained there for several hours. This was presumably when final egg maturation occurred. The evidence suggests that fish released their eggs and sperm as they rushed toward the surface before dawn.

f. Demonstrated that wild broodfish captured from a PSA can be held for several days, will respond to hormonal treatments, will complete egg maturation, and can be manually strip-spawned. This achievement represents the World's first hormonally induced spawning of bonefish.

3) Applied the information on the reproductive process from wild fish to captive breeding protocols

a. Successfully adapted information on hormonal treatments in field studies for spawning in the controlled culture systems at HBOI.

b. Studied the response of bonefish broodstock to multiple hormone treatments intended to induce spawning.

c. Male fish routinely attained reproductive maturity in the controlled breeding systems at HBOI. Determined methods for conditioning and full-term maturation of preovulatory eggs and regularly applied them in wild bonefish females in the controlled breeding populations.

d. Developed a nutritionally enhanced gel-type conditioning diet for broodfish that promotes egg development using information from the field studies.

e. Achieved captive spawns of bonefish, a World first using environmentally controlled conditioning and hormonal induction of ovulation and spawning behavior in spawns with *A. goreensis*.

4) Characterized embryonic development, and larval development and behavior

a. Achieved embryonic and larval growth on multiple occasions:

i. Documented that bonefish embryos produced from field spawning events underwent complete embryonic development and hatched 24-26 hours after fertilization.

ii. Observed that newly hatched larvae utilized internal yolk and oil reserves to support initial larval growth and development of jaws, digestive system, muscles, eyes and other organ systems.

iii. Larvae from two of the captive spawns (see 4.c.) were successfully reared for just short of two weeks (13 days), representing the longest duration of larval rearing of bonefish.

b. Began to understand dietary needs of larval bonefish:

i. Successfully transported larvae to HBOI from the M/Y *Albula* and reared to nearly 10 days in age. Multiple manufactured diets and diets compounded at HBOI were offered to the larvae and feeding was observed but no diet supported long term development.

ii. Demonstrated that bonefish leptocephalus larvae use a lipid class, tied to buoyancy and energy reserves, to sustain their development during the first few days, when they rely on endogenous nutrition.

iii. Larvae from two of the captive spawns were used to test several different diets including a diet used by Japanese researchers to grow eel through to settled juveniles. Although feeding behavior was observed none lived past 13 days.

5) Combined knowledge of larval biology to better model larval dispersal, informing connectivity between spawning and juvenile locations

   a. Demonstrated that bonefish leptocephalus larvae use a lipid class tied to buoyancy which should effect their early larval movements and behavior. Larvae initially have an upright rather than horizontal posture just as is seen in larval eels.

   b. Developed larval transport models based on newly acquired oceanographic information on spawning habitat that inferred the drift and survivorship of larvae during their 41-71 day larval period. The models also determined likely larval settlement locations. Very dramatic differences in annual settlement success and locations were found in models created for different cohorts of larvae produced from the Abaco PSA site. Widespread utilization of these models across the geographic range of bonefish have the potential to identify critical spawning sites and to evaluate interannual variability in juvenile abundance at different locations.

   c. Provided information on the feeding and growth of juvenile bonefish in captivity, as well as benthic habitat characteristics selected by the juveniles.

   d. Identified potential juvenile habitat sites in the Florida Keys based on seasonal seine surveys. The BRRP survey data indicate that shallow, protected sandy habitats with little seagrass or macroalgae serve as nurseries for juvenile bonefish.

6) At the conclusion of the spawning studies at HBOI, broodstock were donated to Sea World, Orlando and the Florida Keys Encounter Aquarium where they will become part of the exhibits for visitors.

While the majority of planned research was achieved, two aspects were partially achieved, one more fully than the other – routine spawning of broodstock in captivity and determining methods to feed and growing the unusual leptocephalus larvae, respectively. To address these remaining objectives, we developed a partnership with the Marine Life Aquarium at the Atlantis Resort in Nassau. Our plans in the final year of the project included setting up temporary spawning tanks near PSAs on nearby islands in the Bahamas, catching and field spawning adult fish, and transporting the embryos to the Aquarium where a larval rearing system would be set up for larval feeding and growth studies. As the spawning work progressed, field personnel also would make more extensive oceanographic measurements of spawning habitat and track adult bonefish on their spawning runs. BTT/HBOI personnel would be temporarily transferred to the spawning and larval culture sites for these endeavors. Logistical planning and supply were completed for this planned work. Unfortunately, travel restrictions due to the Covid-19 pandemic coincided with the cessation of Research Permits being issued by the Bahamian government during 2020 and 2021. A Permit was finally issued in February 2022 just prior to the end of the project performance period, reopening the possibility of doing the critical larval feeding and growth studies, along with additional examination of the larval biology and behavior, in the future.

In addition to the direct research itself summarized above, the BRRP research team produced:

- 12 papers published in the scientific literature, with additional papers in review or in preparation for submission soon.

  -Copies of these articles are presented in the Appendix of this report.

- 15 research presentations and seminars at scientific conferences and universities.

- Three M.S theses and one Ph.D. dissertation
- Postdoctoral training for one individual
- Extensive outreach efforts, including talks to non-technical audiences, articles in print media and their web sites, production of numerous videos posted on-line, and many on-line news media and social media releases. The potential audiences of each of these outreach efforts ranged from hundreds to millions of viewers for a total reach of >40 million via media (i.e., television and print news) outlets, and over 24,000 via social media (i.e., Facebook and Instagram) over the course of the project.

Introduction

Bonefish are an important component of the recreational flats fisheries in the Caribbean and western Atlantic. The annual regional economic impact for these fisheries is high: US$465 million in the Florida Keys (Fedler 2013); part of a US$1 billion fishery in the Florida Everglades; more than US$161 million in the Bahamas (Fedler 2019); and more than US$50 million in Belize. Flats fisheries are composed of anglers fishing on their own, as well as anglers who rely on professional guides, and attract anglers from around the world. In the Bahamas, Belize (Fedler 2014), and other Caribbean locations, the fishery can provide the majority of economic impact for many communities. In addition, the fishery is culturally important: the occupation of professional guide is passed along generations—in some instances, three generations of a family are active guides.

In locations with an established flats fishery, bonefish are mostly catch and release, either by law—as in the Florida Keys and Belize—or as part of an evolving fishing ethos. In other locations, such as Cuba, the flats fishery occurs in protected areas where the species are catch and release only—outside these protected areas bonefish are harvested, in some cases intensively. In all locations, enforcement of regulations remains a challenge. Despite their economic importance, until recently there were large gaps in our knowledge of bonefish ecology.

The limited knowledge is especially disconcerting because an International Union for Conservation of Nature assessment classified bonefish as near threatened due to steep population declines in some locations from intense harvest of adults in recent decades, from the loss of larvae produced in those areas that can drift for several months to maintain populations in other regions, and from habitat loss/degradation that affects nursery habitats for juveniles and feeding habitats for adults (Adams et al. 2012).

Bonefish population in the Florida Keys declined dramatically from the 1990s through the 2010s with fishery participants estimating that the bonefish population declined >90% over the past 25 years (Rehage et al. 2019). The reasons for the bonefish population decline in the Florida Keys are unclear, but numerous factors affecting the production or recruitment of juveniles that supply the adult fishery are suspected. Small juveniles have not been found in the Keys despite intensive field survey efforts, and interviews with long-time fishing guides in the Florida Keys suggest a decline in sightings of juveniles over the past 15 years. The most likely causes are both local (declines in water quality and associated changes in habitat and prey, habitat loss and degradation) and regional from the loss of larvae produced by spawning aggregations in other areas (Adams et al. 2014).

The Bonefish Reproduction Research Program (BRRP) was developed as a partnership between the Bonefish and Tarpon Trust (BTT) and the National Fish and Wildlife Foundation (NFWF) to help assess possible mechanisms to help recover and maintain the population of bonefish in the Florida Keys, and to ultimately provide tools that can be used in other regions where the populations are declining. Intense field and aquaculture investigations were conducted by this program to understand many of the biological, physiological and ecological characteristics associated with reproduction and subsequent larval recruitment into the juvenile life stage. Developing this comprehensive understanding of bonefish reproduction and factors affecting the survival, growth and dispersal of early life stages (eggs, larvae and juveniles), can help guide the development of management plans that protect valuable spawning areas, as well as identifying, restoring and managing optimal nursery habitats.

The Bonefish Restoration Research Project has resulted in a substantial increase in the understanding of Bonefish biology, and in particular the reproductive biology related to spawning

and larval life history. The overall approach was to study both wild bonefish in The Bahamas, and captive bonefish at HBOI (Figure 1). Overall, the BRRP has substantially filled in these knowledge gaps and has made a quantum leap in advancing the understanding of bonefish biology (Figure 2), particularly in the realm of adult reproductive biology, and larval and juvenile life history.

Wild Bonefish Reproductive Biology and Life History

Assessment of reproductive physiology of Bonefish across the Bahamas

We characterized ovarian development and reproductive hormones of bonefish *A. vulpes* on a wide geographic scale. 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; and (2) characterize reproductive phases for female bonefish at flats and PSAs during the spawning season. 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 location is relatively pristine, contains substantial flats habitat, and has a single identified PSA.

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 (Figure 3). 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) (Figure 3). Individuals on the tidal flats were captured using a 50 m x 1 m beach seine with a 2.5 cm mesh. All individuals collected were kept

for 3-5 minutes in large plastic, floating containers modified with holes to allow adequate water exchange. Fish in the PSA were captured via hook and line. 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. 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. 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 vacutainerTM. 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, 57 females (ranging from 373 to 640 mm FL) were sampled from flats (n =37 fish) and PSAs (n =20 fish) during the spawning season. 17β-estradiol (E2) and testosterone (T) concentrations were quantified via enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical Company, USA). The plasma sample 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). 50 µl 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.

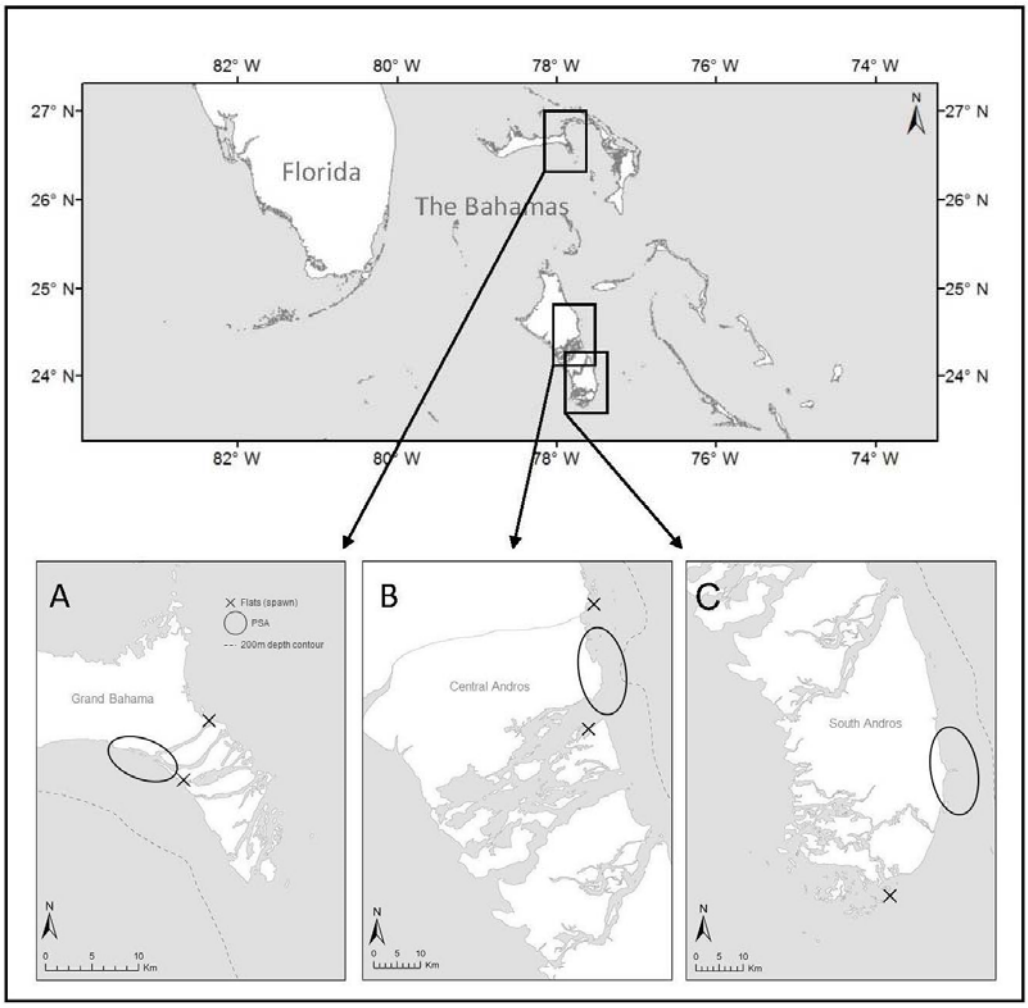


Figure 3. Map showing sampling locations across three islands in The Bahamas, and the respective flats and pre-spawn aggregation (PSA) habitat sampled within each. Flats habitat was sampled during both months and are labeled as Flats (spawn). All islands were sampled during the full moon of a spawning 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.

Across all islands, significant differences in mean concentration of 17ß-estradiol were observed. Comparison between flats and PSAs within islands revealed that mean concentrations of 17ß-estradiol were significantly higher at the PSA compared to flats habitat (Figure 4). Testosterone levels were not significantly different when compared across all islands. Within each island, testosterone concentrations were significantly higher in fish sampled from the PSA

compared to those sampled along flats (Figure 4). LH levels did not significantly differ when compared across all islands. However, within each island, LH levels were significantly higher at PSAs compared to flats habitat (Figure 4).

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,

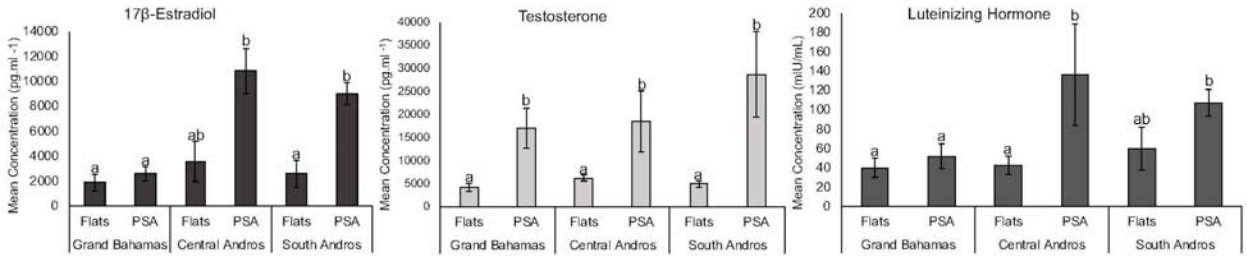


Figure 4. Spatial variation in 17β-estradiol, testosterone, and luteinizing hormone concentrations (mean ± SEM) for bonefish (Albula spp.) sampled across three islands (Grand Bahama, Central Andros, and South Andros, The Bahamas) and two habitat types (Flats and PSA) within each island. Black letters indicate significant differences (Two-way ANOVA).

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, samples were pooled based on assigned reproductive classification. Significant differences in mean 17β-estradiol were found among reproductive phases exhibited by bonefish sampled along flats. This difference was driven by significantly lower 17β-estradiol levels in "immature" and "regressing" fish compared to those "developing" or "spawning capable" ones (Figure 5). Mean concentration for testosterone did not significantly differ by reproductive phase (Figure 5).

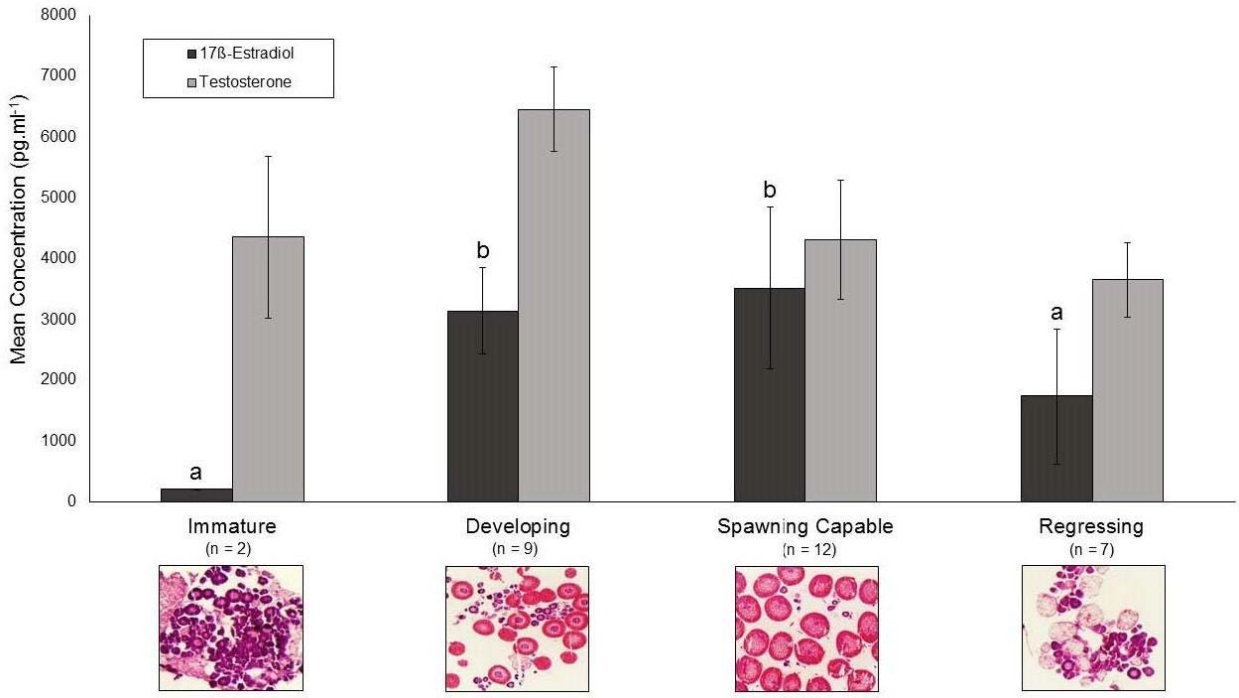


Figure 5. Concentrations of 17β-estradiol and testosterone (mean ± SEM) for each reproductive phase observed in flats from female bonefish (*Albula* spp.) across all islands (Grand Bahama, South Andros, and Central Andros, The Bahamas). Letters indicate significant differences in mean concentration of 17β-estradiol across phases (One-way ANOVA). No significant differences were observed for testosterone.

Hormone Cycle Profiles in Adult *Albula vulpes*

We developed a reproductive hormone profile of wild bonefish captured in Bahamas and spatially characterized bonefish reproduction using egg development metrics. The main objective was to provide a baseline of what hormone levels should look like in wild population of bonefish. The application for this dataset was two-fold. First, to provide the project with natural (expected) reproductive hormone levels in successful, wild spawning fish. We were able to compare these values to those of our captive fish to potentially identify whether reproductive dysfunction is occurring in captivity. Second, as a baseline for analyzing the reproductive viability of Florida bonefish, which may experience a multitude of stressors (pharmaceuticals, pollution, etc.) that

hinder spawning. This study helped understand the developmental purpose of PSAs and provide the bonefish restoration project with egg development data from wild, spawning fish as a baseline for captive rearing.

Blood was collected from female bonefish at Grand Bahama Island before, during, and after the full moons – bonefish are most frequently documented in PSAs near full moons. Fish were sampled both along the Flats (non-spawning habitat) and in the PSA. It is notable that PSAs occur in locations different from non-spawning home ranges of bonefish. Blood was centrifuged to separate out plasma. Plasma was analyzed via ELISA (Enzyme linked immunosorbent analysis) to quantify Estradiol (estrogen) and Testosterone levels in females. Both hormones are responsible for the successful development of eggs. Low levels of either hormone relative to the norm are indicators of reproductive dysfunction. Eggs were also collected from both Flats and PSA sampled fish. After being prepared for analysis via histology, eggs were measured and quantified by type (developmental state) using a microscope equipped with a digital camera and measuring software.

Bonefish ovaries exhibited characteristics that suggest they spawn multiple times during the spawning season. These characteristics are seen below with the occurrence of three clearly different developmental stages of eggs (Figure 6). Bonefish eggs were also significantly larger (591 ± 134 μm) at the PSA than those collected on the flats (556 ± 129 μm), indicating that fish are generally more prepared to spawn at the PSA than those residing on the flats. No hydrated eggs (fully mature) were found at the PSA indicating that final maturation is occurring somewhere offshore during the spawning run.

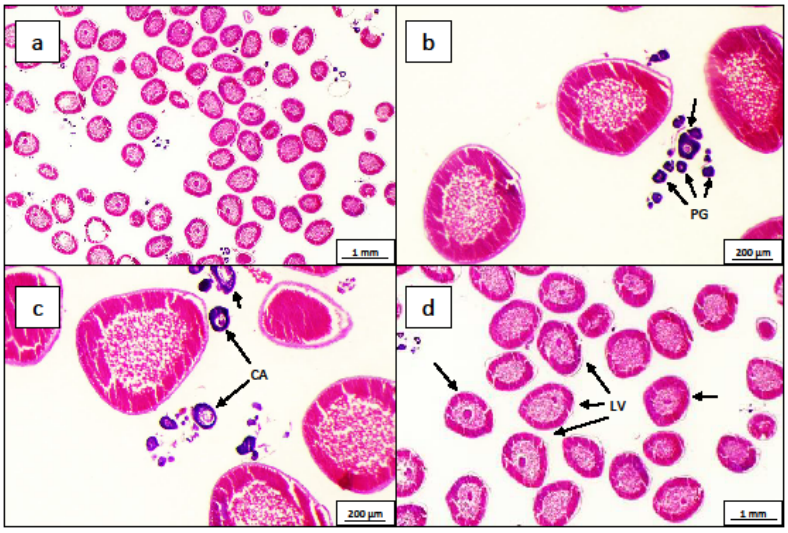


Figure 6. Micrographs of a histologically sectioned oocytes sample collected from a wild female bonefish (*Albula vulpes*) on a shallow water flat during full moon (April 2017). (a) three different developmental oocyte stages are present: (b) primary growth (PG), (c) cortical alveolar (CA), and (d) late vitellogenic (LV). Oocyte samples collected at the pre-spawn aggregation (PSA) were visually similar to flats samples.

Furthermore, the frequency of late vitellogenic eggs (developmental stage closest to maturation) was significantly higher in fish sampled at the PSA than those on the Flats (Figure 7). The high variability of oocyte frequencies on the flats indicates that fish are at different levels of development and likely make their way to spawning grounds at different times. Estradiol, the hormone primarily responsible for vitellogenesis (yolking of egg) did not significantly change

from Flats to PSA fish. Significant increase in T levels between Flats and PSA is an indication that final maturation is very close to occurring (Figure 8).

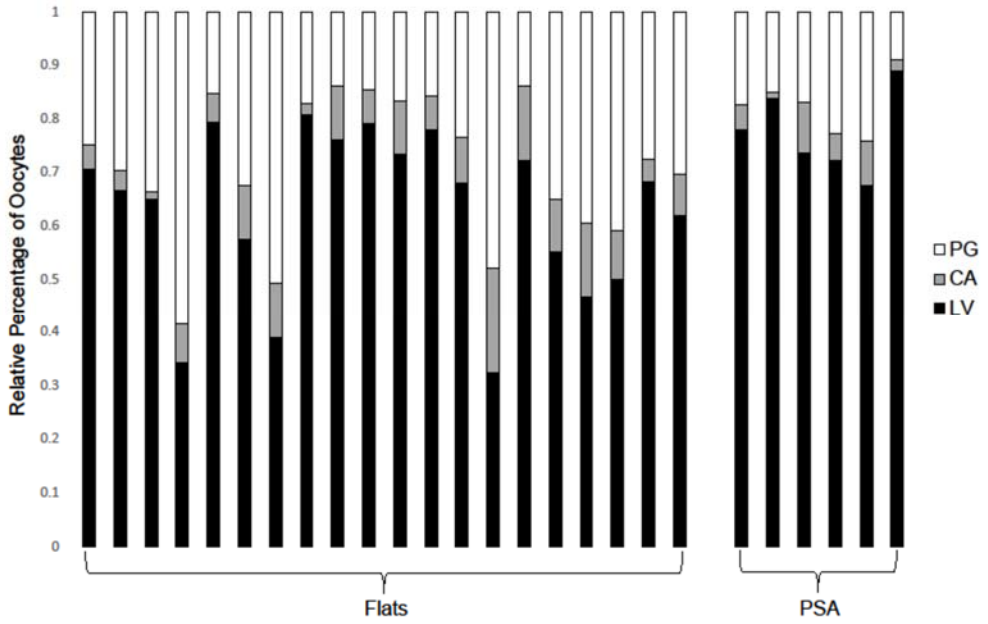


Figure 7. Relative Percentage of Each Egg (oocyte) Stage Present

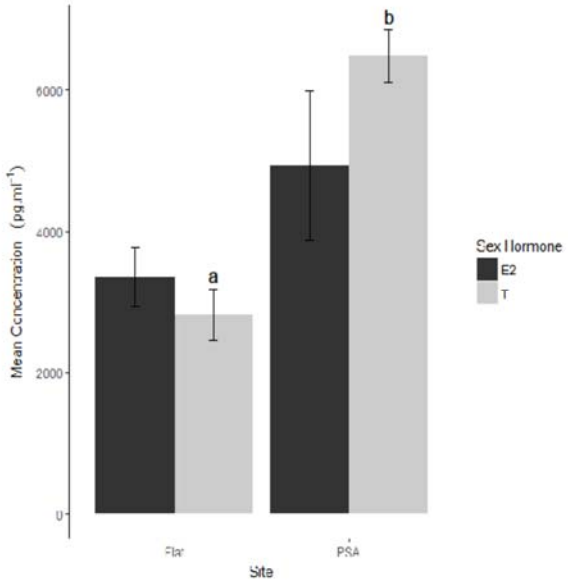


Figure 8. Spatial Hormone Level Comparison

Spawning of Bonefish

"Wild" Bonefish Spawning

Spawning trials at Grand Bahama 2016 and South Andros 2016-2017 spawning season

We attempted several different hormone injections to induce captive spawning in naturally conditioned fish captured from pre-spawning aggregations (PSAs) during the natural reproductive season on Grand Bahama and South Andros. Bonefish males and females were collected by hook-and-line in April of 2016 near East End Lodge on Grand Bahama, and in January 2017 at High Point Cay on South Andros. The fish captured between January 12, 2017 and January 14, 2017 on South Andros included three males that were injected with 500 IU/Kg HCG, and nine females that received different trials of hormone injections to induce ovulation (Table 1 and 2). We did not observe ovulation, but progression of development was observed following some injections based on observations of asymmetry in the oil droplet portion of the yolk material. We now know that this effect is due to migration of the germinal vesical. This characteristic of bonefish eggs during development was not commonly known previously and is not characteristic of most marine fish.

Table 1. Results of hormone injections and spawning attempts from bonefish captured during April 2016 near East End lodge on Grand Bahama.

Fish #	4/21/2016	4/22/2016	4/23/2016	Notes
BAP3985	1.5 ml Ovaprim	1.5 ml Ovaprim	1.5 ml Ovaprim	No Ovulation
BAP3450		3.0 ml Ovaprim	3.0 ml Ovaprim	No Ovulation
BAP3981		captured		Strip Spawn Attempt No Fertilization - Eggs don't appear to have ovulated
BAP3979			captured	Strip Spawn Attempt No Fertilization - Eggs don't appear to have ovulated

Table 2. Different hormone combinations injected into bonefish females captured at South Andros in January 2017, in attempts to induce spawning. Injections included human chronic gonadotropins (HCG), Luteinizing Hormone-Releasing Hormone Analog (LHRHa), Ovaprim, carp pituitary extract (CPE), and Dihydroprogesterone (DHP).

Captured 1/12/17 AM, returned to tanks, 1.5 hour trip, cannulated/injected at 1730 Second injection 1/13/17 at 1800						
Fish ID	Sex	FL	Girth	Initial Hormone/Dose	Resolving Dose	Result
BAA1137	M	389	240	HCG 500 IU/kg	HCG 500	Mortality 1/15 0600
BAA1138	F	430	280	HCG 1000 IU/kg	HCG 1000	No ovulation
BAA1139	F	487	290	Control	HCG 1000	"
BAA1140	F	485	290	LHRHa 75 ug/kg	HCG 1000	"
BAA1141	F	415	250	CPE 8 mg/kg	HCG 1000	Eggs resorbing? Maturing?

Captured 1/13/17 AM, returned to tanks, 0.5 hour trip, cannulated/injected at 1730, no 2nd dose, hand checked 1/15 0630, released						
Fish ID	Sex	FL	Girth	Initial Hormone/Dose		Result
BAA1142	M	419	220	HCG 500 IU/kg		
BAA1143	F	440	255	Ovaprim 1ml/kg		Mortality 1/14 0600
BAA1144	M	377	220	HCG 500 IU/kg		
BAA1145	F	446	260	LHRHa 150 ug/kg		No ovulation
BAA1146	F	461	280	Ovaprim 1.5 ml/kg		Mortality 1/14 0600
BAA1147	F	465	270	Ovaprim 1 ml/kg		No ovulation
BAA1148	F	570	345	control		"

Spawning trials at Cape Eleuthera Institute (CEI) 2017-2018 spawning season

In collaboration with Cape Eleuthera Institute (Figure 9) in the Bahamas, we attempted several different hormone injections to induce captive spawning in naturally conditioned fish captured from PSA during the spawning season. Bonefish males and females were collected by hook-and-line at No Name Cut, a known PSA site during the full moon periods of September 2017, November 2017, December 2017, and January 2018, of the 2017-2018 spawning season.

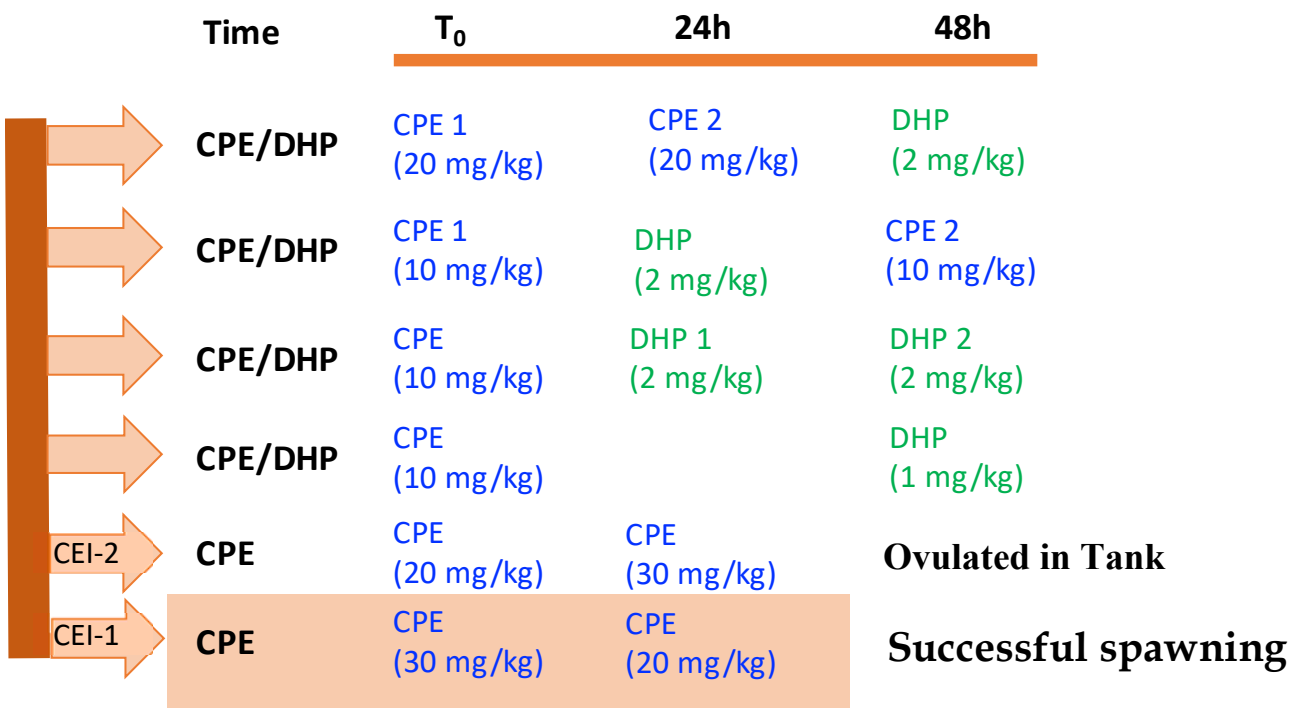
Figure 9. Cape Eleuthera Aquaculture facility where captive bonefish were held under natural temperature and light

Reproductive hormones were administered to fish collected during the full moons in December and January, with initial injections provided during the evening after capture. Second and third injections were administered 24 and 48 hours later. Three males had were producing milt when they were captured, and were injected with HCG hormone to maintain sperm production. Six females collected during these two periods received a combination of hormone injections (CPE and DHP) and the eggs were cannulated and checked for maturation and size at 24h and 48h (Table 3). The progression of development was assessed by the increase in the egg diameter and the migration of the germinal vesicle (Figure 10d). During the spawning attempt in January 2018, two of these females ovulated (CEI-1 and CEI-2). One ovulated in the tank (CEI-2) in between hormone injections and routine checks, so the eggs were not able to be collected for fertilization. The other (CEI-1) was successfully strip-spawned following the second injection

of CPE, starting with a high dose (30 mg/kg) and decreasing the dose after 24h to 20 mg/kg.

This represents the World's first successful artificial spawning of bonefish.

Table 3. The different hormone combinations injected to bonefish females to induce spawning were carp pituitary extract (CPE), and Dihydroprogesterone (DHP).



Induction of spawning during M/Y Albula research cruise trips 2018-2019

Initial attempts to induce spawning in wild bonefish focused on the development of procedures to assess reproductive condition of females prior to spawning, and identification of candidate hormones responsible for triggering the final stages of oocyte development (Mejri et al., 2019). In our previous work, we demonstrated that advancement of gonad development is possible in the laboratory using a combination of environmental manipulation and carp pituitary extract (CPE) injections, when oocytes are ≤ 700 µm, but no ovulation or spawning occurred (Mejri et al., 2019). The BRRP was successful at inducing ovulation and spawning of multiple *A.*

vulpes aboard the M/Y *Albula* in the Bahamas. The specific objectives of this study were to: 1) capture reproductively mature fish from pre-spawning aggregations, 2) induce final oocyte maturation and ovulation through administration of CPE, and 3) describe and document embryonic and early larval development.

Sexually mature *A. vulpes* (n = 18 females; n= 25 males) were collected by hook and line from November 2018 to March 2019 from pre-spawning aggregations at Great Abaco, and Bimini, and on the flats at Long Island, Chub Cay, The Bahamas. Precise locations are not being published in the interest of PSA conservation.

Broodstock collected in Great Abaco (n = 4 females; n = 8 males; Fish designation = ALB; FL = 405-480 mm) were held in 1500 L circular tanks under ambient light and supplied with flow-through sea-water. Cannulated oocytes of > 700 μm in diameter (Mejri et al., 2019) was the criterion used to select candidate females for spawning trials. Presence of freely-flowing milt was used to select males.

Three females received initial intraperitoneal injections of 20 mg kg^{-1} of CPE (Stoller Fisheries, Spirit Lake, IA) in early evening within 8-10 hours of capture (Table 4). A second 20 mg kg^{-1} CPE injection was administrated 24 h later. The last female received only a single injection of CPE (60 mg kg^{-1}) to test the effect of a larger single dose on final oocyte maturation and ovulation (Table 4). Because all males released sperm upon abdominal pressure, no hormonal injections were administrated. All fish were monitored at 4-6 h intervals until signs of ovulation had occurred, observable by the presence of a significantly distended abdomen. For spawning, eggs and sperm were extruded into separate bowls by gentle massage of the abdomen. Sperm was then added to the eggs and gently mixed with a feather. Ambient seawater was then

added to activate sperm and promote fertilization. Fertilized eggs were left to rest for 5 minutes before being rinsed three times with ambient sea water and incubated.

Fertilized eggs were incubated in two 6 L polycarbonate containers supplied with filtered ambient seawater which were placed in a static 100 L water bath to maintain temperature (24.8 ± 1°C). A small air stone in each container provided gentle aeration, 25% partial water changes were conducted every hour with a 50% water change every 6 h during embryogenesis. After hatching, 50% water changes were conducted every 4 h and dead eggs and larvae removed from the bottom of the incubation container to maintain water quality

Table 4. Summary of oocyte development of *A. vulpes* females from Abaco (ALB-1, ALB-2, ALB3, and ALB-4), The Bahamas, showing oocyte diameter (+/-S.D.), and relative percentages of oocytes in different developmental stages (DS) at initial sampling (T_0), 24 (T_{24}), and Time strip spawned (T_X) hours following carp pituitary extract (cPE) injections. ALB-2 received only a single injection of CPE at 60 mg kg^{-1} whereas the other three received two intraperitoneal injections of 20 mg kg^{-1} of CPE spaced 24 hours apart. LV: late vitellogenic stage, GVM: germinal vesical migration, and GVBD: germinal vesical breakdown. Symbol (-) in the table indicates no data was collected or the absence of sampling at that time. Oocyte diameter measure represents the mean ± SD from 35 to 37 oocytes.

Fish ID	**Oocyte diameter (µm)**			**Relative percentages (%) of DS**						**Time spawned**
	Hours post CPE injection			T_0			T_{24}			
	T_0	T_{24}	T_X	LV	GVM	GVBD	LV	GVM	GVBD	
ALB-1	701.1 ± 50.8	926.7 ± 42.1	980.1 ± 97.4	76	16	8	28	62	10	33h30min post T_0
			*measure at ~ 33 post T_0							-Strip spawn
ALB-2	988.5 ± 50.8	-	-	0	61	39	-	-	-	12h post T_0
										- Strip spawn
ALB-3	938.2 ± 65.5	1012.3 ± 12.8	-	0	90	10	0	0	100	≥ 28h post T_0- released eggs in tank
ALB-4	704.9 ± 46.7	961.5 ± 41.45	-	80	20	0	33	63	3	≥ 28h post T_0- released eggs in tank

Final Egg Maturation and Early Embryonic Development from Spawns at CEI and on the M/Y Albula Cruises

Photographs of cannulated oocytes and developing embryos were taken every hour from fertilization until hatching, using 1.3 MP Dino-Lite Edge microscope (Dino-Lite, Torrance, CA). Larvae were photographed initially at two-hour intervals (hatching i.e. 0 h, 2 h, 4 h, and 6 h post-hatch [hph]), after which sampling was reduced to every four hours, until no live larvae remained.

Females captured at the PSA sites all contained oocytes in advanced stages of development and completed their maturation in response to CPE administration. Oocytes removed from the ovaries within hours after capture but prior to CPE injection had a uniformly smooth, opaque, orange-yellow appearance (Figure 10a). Almost all oocytes were in the late vitellogenic (LV) stage of development (Figure 10b-c) with mean diameters of 713-840 µm (Table 3). Oocytes collected after 24 h, after the first CPE injection, had increased in size to about 900µm (Table 3), 76-89% were undergoing germinal vesicle (nucleus) migration (GVM) toward the periphery of the oocyte (Figure 10d) and 6-11% had begun breakdown of the germinal vesicle membrane (germinal vesicle breakdown-GVBD) (Figure 10e). By 12 hours after the second injection, CEI-1 had a very distended abdomen, ovulation had occurred, and fully hydrated eggs were easily released with light abdominal massage. Eggs increased to a mean diameter of 1.1 mm, with the contents separating into transparent yolk proteins and numerous oil droplets (Figure 10f-g). CEI-2, ALB-3 and ALB-4 were not manually spawned but are believed to have released eggs in the tank overnight and were unrecoverable. Interestingly, CEI-1, CEI-2, ALB-1, ALB-3 and ALB-4, which received two intraperitoneal injections of 20 mg kg^{-1} of CPE spaced 24 h apart and ALB-2, which is the only fish to receive a single dose of CPE at 60 mg kg^{-1}, all resulted in oocytes completing final maturation, hydration, and ovulation.

Eggs prior to fertilization and immediately after fertilization had a mean diameter of 1.2 ± 0.47 mm, with the yolk having a mean diameter of 1.05 ± 0.23 mm. The dense array of oil droplets on the upper surface of the egg obscured visual examination of early embryonic development, but a disc of cells could be seen on the lateral surface of the egg by around 8 hours post-fertilization (hpf). Oil droplet coalescence did not begin until 21 hpf and was not complete for several embryos prior to hatching, which occurred at 25-26 hpf. The zygote and cleavage stages lasted 1-2 h, with blastula (Figure 10h), gastrula (Figure 10i) and segmentation stages (Figure 10j) each persisting for 6-9 h. The pharyngula stage (Figure 10k) occurred during the final 2-4 h prior to hatching. Larvae hatched from the eggs 24-26 hours after fertilization.

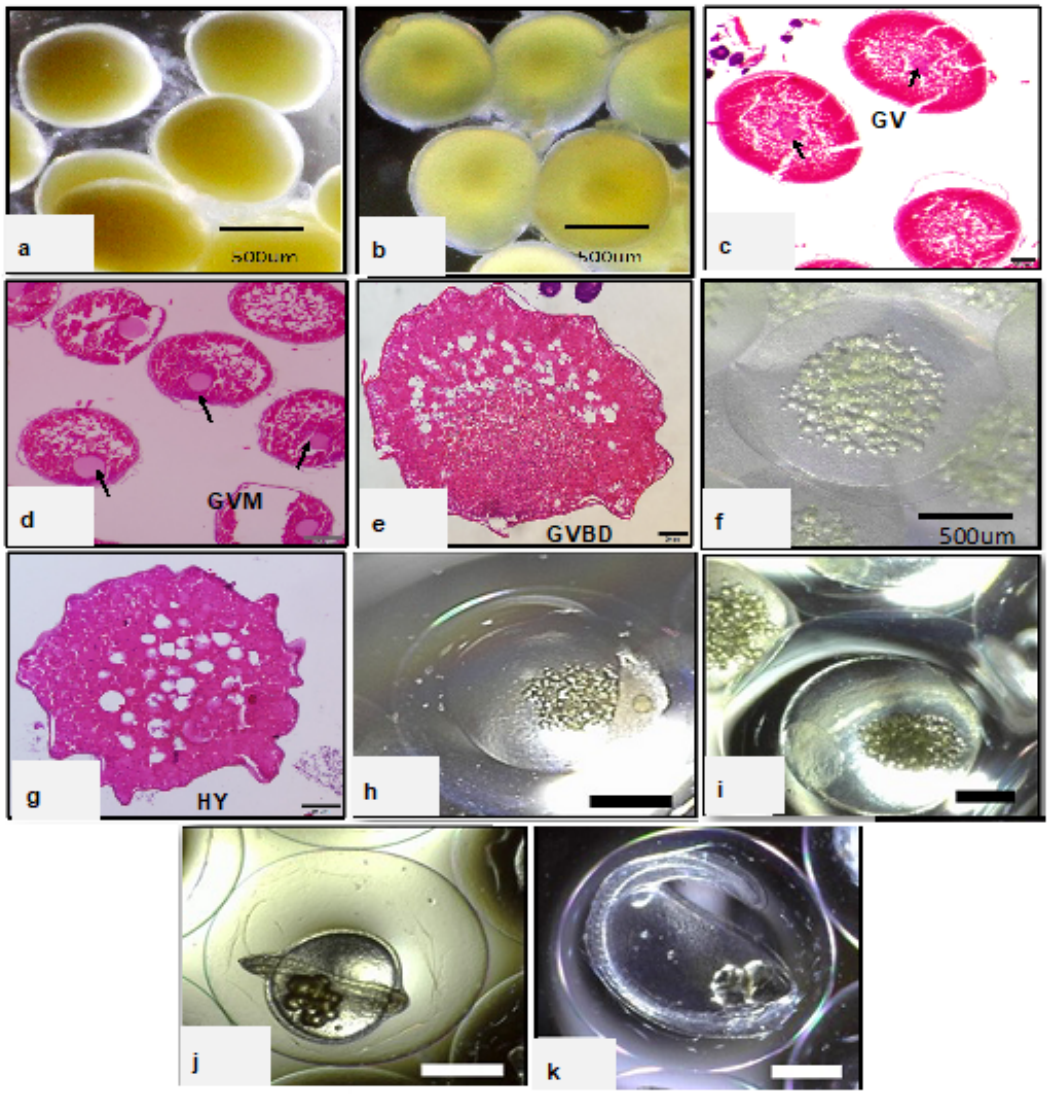


Figure 10. Images and micrographs of histologically sectioned oocytes and embryonic development of *A.vulpes*. Image a) shows smooth, opaque orange-yellow appearance of an oocyte, b)-c) shows the germinal vesicle (GV) central position in oocytes before the first carp pituitary extract (cPE) intraperitoneal injection, d) shows the germinal vesicle migration (GVM) in oocytes 24h post cPE injection, e) shows a germinal vesicle breakdown (GVBD), f)-g) shows a hydrated (HY) oocyte, ready to be fertilized with the contents separating into transparent yolk proteins and numerous oil droplets, h) embryo in the blastula stage (8 hours post fertilization), i) embryo in the gastrula stage (11 hours post fertilization), j) Embryo in the segmentation stage (21 hours post fertilization), k) embryo in the pharyngula stage (24 hours post fertilization). Scale bar for images h, i, j, and k = 0.5 mm.

Captive Spawning at Harbor Branch Oceanographic Institute

Captive Broodstock Acquisition

- ***Bonefish Broodstock collected - A. vulpes and A. goreensis***

Total number of broodfish in culture at Harbor Branch Oceanographic Institute (HBOI) over the life of the project was: 78 *Albula vulpes* and 71 *A. goreensis*. Additionally, we had 56 juvenile *A. goreensis* that were tank raised from post-settled fingerlings to brood size.

- ***Broodstock from the Lower and Middle Florida Keys, FL – A. vulpes***

A large effort for broodstock collection was coordinated with the assistance of Carl Navarre and several bonefish guides in the area around USF's Keys Marine Laboratory. Fishing guides who captured fish delivered them to KML tanks and KML staff cared for them until our staff traveled there to transport them to HBOI. This effort captured a total of 52 *A. vulpes* broodstock, all over 20 inches in length. Three of these fish delivered to KML by guides died under KML staff care, likely due to stress related to hook and line capture and transport to KML, therefore a total of 49 broodfish were transported to HBOI.

- ***Broodstock from the Key Biscayne area, FL (Albula vulpes)***

Through the effort of a fishing guide and HBOI anglers, a group of 20 *A. vulpes* broodfish over 20 inches in length were captured and stocked into one of the HBOI spawning conditioning systems on Sept 27, 2018 and October 2, 2018 to begin conditioning in preparation for hormone response trials. None were showing signs of gonadal development based on cannulation at the time of capture.

- ***Natural Photo-Thermal Cycled Captive Broodstock Volitional Spawning Trial***

A total of 46 *A. goreensis*, were collected with the help of fishers during multiple collection trips to Sandsprit Park in Stuart, FL between November, 2017 and February, 2018. Forty-five of these fish were initially stocked in the large capacity, 20,000 gallon, conditioning/holding system in the HBOI Aquaculture Park. After the initial stocking an additional 26 *A. vulpes* were added to the tank beginning in August 2018 and an additional 31 *A. vulpes* were added in November 2019. These *A. goreensis* and *A. vulpes* were maintained in the large tank that allowed us to assess whether mass schooling behaviors, natural light and temperature could induce maturation and volitional spawning with no other intervention (i.e., hormone injections). The only interaction these animals received were daily wellness checks and feedings. They were left in this system for three spawning seasons. This method was also replicated at the Atlantis Aquarium, Nassau, Bahamas, to attempt to repeat volitional spawning, which was incidentally seen there with *A. vulpes* several years ago.

Captive Broodstock Diets

Initially, broodfish were fed a diet of frozen shrimp, krill, clams, and caplin. They primarily only accepted the frozen shrimp. Although they were well fed on the shrimp diet and were growing well, we noticed during necropsies of fish that died for various reasons had excessive adipose fatty tissue. Also, nutritional analysis of the frozen shrimp indicated that there were missing fatty acids that likely would be needed by the females to produce viable eggs that would translate into health larvae. Therefore, we designed a gelatin-based diet (Gel Diet) formulation that had fatty acid profiles similar to that seen in wild fish. The gel diet was not accepted as well as the shrimp initially even though it had a soft texture like shrimp. However,

with gradual weaning from shrimp and onto the gel diet for two or so weeks the fish began accepting it well and did thrive on the new diet as evidenced by good growth measured during spawning maturity checks. The diet should be fed every other day at 5% body weight divided into four feeding per day.

Gel Diet Recipe:

- 500 grams frozen krill
- 500 Grams INVE Fish Breed-M Powdered Diet
- 117 grams unflavored pork skin gelatin
- 800 ml scalding water

Directions

All ingredients should be mixed in a high torque blender until uniformly smooth with no clumps and poured into foil sheet pans in a ½" to ¾" inch deep layer. The pans containing the mixture need to be chilled in a refrigerator for 2-3 hours before cutting the gelatin diet into cubes that are appropriately sized for the animals being fed. The cubed gel diet can be frozen until needed. It is recommended that 1 weeks' rations of feed be made at a time to avoid freezer burn.

Hormone induction and Broodstock conditioning

Multiple hormonal maturation/induction treatments were tested for induction of gonadal maturation and induction of ovulation throughout the project. Ultimately, this effort resulted in three volitional spawning events, two in October 2020 (October 5th and 6th), and one on February 12, 2022.

Prior spawning attempts indicated that female *A. vulpes* and *A. goreensis* showed positive signs of gonadal maturation as a result of photo-thermal cycling in conjunction with Carp Pituitary Extract (CPE) treatment but ultimately did not spawn.

Salmon Pituitary Extract (SPE), the hormone used by the Japanese to induce spawning in eels, also showed positive results for gonadal maturation in female *A. goreensis* (Figure 11). During the initial years of the project SPE was not available due to lack of supply in the USA. However, a new supplier came on line with the product and we had the opportunity to use it for our trials. Ultimately SPE paired with salmon gonadotropic hormone analog (sGNRHa) resulted in the successful spawns (see detailed discussion below).

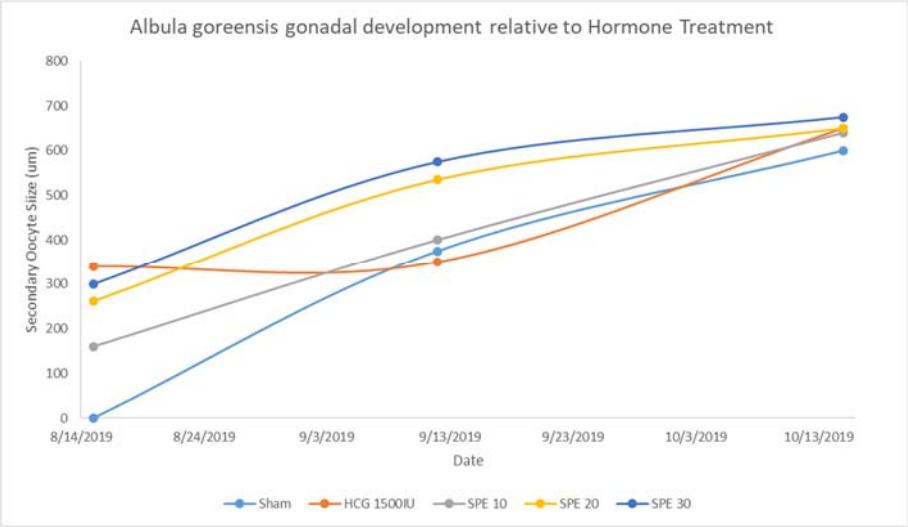


Figure 11. Maturation of *A. goreensis* as a result of photo-thermal cycling and hormone stimulation with SPE.

Male *A. goreensis* that were in the tanks with the *A. goreensis* females mentioned above were all producing sperm that could be easily expressed by pressure on the abdomen (i.e., running ripe) regardless of hormone treatment (Figure 12). Subsequently, only HCG and sham injections were used for males.

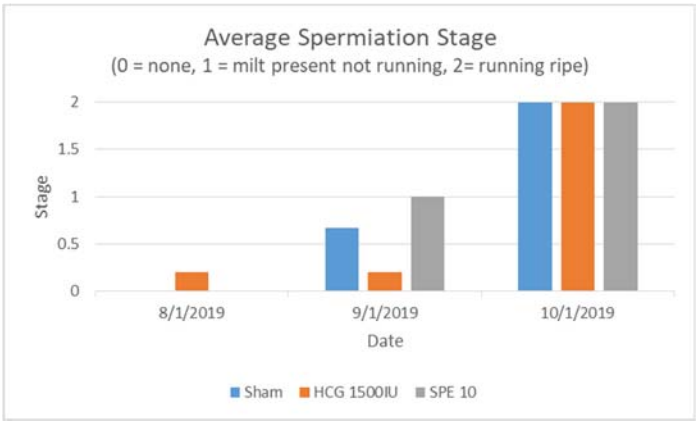


Figure 12. Response of male *A. goreensis* relative to photo-thermal cycling and hormone treatments.

HCG injections at 1000 IU/Kg consistently result in at least 6 months of sperm production before they stop running with milt.

The first group of *A. vulpes* that received the SPE treatments was the group from Biscayne Bay (see above). On January 23, 2019 we began monthly treatments with SPE at 20 mg/Kg and Human Chorionic Gonadotropin (HCG) at 1000 IM/Kg to observe for stimulation of maturation. The treatments were repeated monthly in February and March 2019 with no apparent development, so the group was cycled back into summer conditions and subsequently cycled back to winter for a second maturation/spawning attempt that began in September 2019. During the initial sampling, one female had eggs present in the cannula sample. All animals received an initial SPE treatment of either 20 or 40 mg/Kg, HCG at 1500 IU/Kg, or sham injections with bacteriostatic water as the control group. As of the first monthly post-treatment sample in October 2019, four additional females were noted with small eggs (100-300 microns) present in the cannulation sample. No males were expressing free flowing sperm.

We applied similar techniques using subsequent groups of fish but increasing the dosages of SPE or comparing CPE and SPE. Each time we saw good development of the ovaries but low spawns resulted. The mixture that was significantly verified to worked ultimately (see below) was SPE at 80 mg/Kg monthly followed by a dose of 75 ug/Kg sGNRHa when the eggs reached appropriate size.

On October 5 and 6, 2020, the fish spawn volitionally in the tank for the first time, resulting in the World's first controlled spawns in captivity. In addition, a third volitional spawn happened on occurred February 9, 2022.

Captive spawns- October 5 and 6, 2020

- ***Worlds first successful artificial spawn of captive Albula spp. through thermal and photoperiod conditioning followed by hormonal induction of ovulation***

Thirty-two *A. goreensis* (23 females and 9 males) that were captured between November 2017 and February 2018 in the Indian River Lagoon near Sandsprit Park, Stuart FL, within the channel of the Saint Lucie Inlet with the help of commercial fishers targeting Pompano *Trachynotus carolinus*. Each broodfish was implanted with a uniquely coded Passive integrated transponder (PIT) tag after completing 30 days of quarantine. These broodfish had been through two nonproductive conditioning/spawning cycle attempts prior to the event of this report. For each spawning attempt the fish were conditioned by manipulating the photoperiod and thermal conditions to simulate the seasonal cycle experienced annually by wild fish. The spring, summer and autumn portions of the conditioning cycle, however, were compressed into a 20-week period prior to the winter spawning period where they were then held at the winter temperature (25℃) and photoperiod (9 h Light:15 h Dark). The winter period, which is the spawning season, was maintained for as long as the fish appeared to be progressing in gonadal maturation based on monthly assessments of condition (i.e., increase in size of oocytes of females and presence of running milt in males).

During the winter period, monthly assessments of spawning readiness were conducted around the time of the full moon of the lunar cycle beginning in July 2020 through October 29, 2020. Fish were captured individually from their conditioning tank and anesthetized in 60-100 mg/L of Tricane-S (Tricaine Methanesulphonate, a.k.a., MS222, Western Chemicals, Ferndale, WA, USA) in buffered "Seawater" (29-32 ppt salinity saltwater well water). A gonadal biopsy was collected from each female for immediate and later measurements of fresh oocyte diameters and later histological classification of the gonadal tissue. Males were palpated to determine

whether milt was present and easily expelled (i.e., running). As each of the females was checked they were given different doses of Salmon Pituitary Extract (80 mg/Kg n=6; 120 mg/Kg n=7; and 160 mg/Kg n=7) via interperitoneal injections or sham (n=3) injections (bacteriostatic water) intermuscularly in the dorsal musculature. The nine males were given either HCG at 1500 IU/Kg (n=7) or a sham (n=2) injection intermuscularly in the dorsal musculature as well. Upon collection and immediate microscopic examination of the ovarian biopsies a subjective decision was made as to whether a given female was approaching spawning readiness based primarily on the presence of ova >600-700 μm in the ovarian biopsy. When the decision was made the target female was ready she was implanted with a slow-release pellet containing 75 μg of salmon Gonadotropin- Releasing Hormone analog (sGNRHa) (Syndel Ovaplant®, Ferndale, Washington, USA). sGNRHa Implants were only administered on October 1 and October 29 for this spawning group.

After assessments were completed the broodfish were stocked into a clean conditioning system at the same water temperature and photoperiod as the conditioning system for volitional spawning. The system was set up so that any spawned eggs would be collected in two egg collection tanks in the outflowing water path. These tanks were large enough to slow the water flow velocity greatly and had fine mesh screens to retain the eggs, allowing water to continue to flow to the filtration system without damaging the eggs. Oxygen was continuously bubbled into the egg collection tank to keep any eggs collected alive and developing.

Based on wild spawning conditions noted in Lombardo et al. 2021, the water temperature in the spawning tank was pulsed during each night to simulate the 2℃ decrease in water temperature experienced by wild fish during their deep dive prior to spawning. Each

temperature pulse was completed over the course of the week of spawning from 25-26℃ to 22-23℃ after hormone injections and/or implantation with sGNRHa then back to 25-26℃.

Video of the surface of the tank was recorded by two security cameras illuminated by integrated infrared LED lights throughout the potential spawning period. Videos were observed afterward for any unusual activity that would correlate with spawning activity in the tanks to determine the likely time of any spawning events that occurred.

- ***Parentage Determinations***

Three methods were employed to assess the parentage of the offspring produced by this spawn to assess which of the hormone treatments applied to the mixed spawning group was successful for inducing the spawns. These methods were; 1) to look for changes in the measurement of the diameters of fresh oocytes taken by direct ovarian biopsy from each female monthly, 2) to look for changes in the measurement of the diameters of histological preparations of preserved oocytes taken by direct ovarian biopsy from each female monthly, 3) and using genetic parentage analysis based on sequencing of DNA samples collected from each broodfish (males and females) and from samples of embryos collected after each spawn to determine which fish contributed to spawning.

Fresh Egg Analysis

During monthly sampling to evaluate readiness photomicrographs of fresh oocytes, ovarian biopsies were taken using an infant gastric feeding tube with the tip cut off and attached to a 3 ml syringe (Figure 13). Photomicrographs were taken using a 1.3 MP Dino-Lite Edge microscope (Dino-Lite, Torrance, CA) and a Dino-Lite MSBL-CDW Backlight Stage in Darkfield mode. Photomicrographs were captured as JPEG files using the DinoCapture 2.0 software for Microsoft Windows 10. The DinoCapture software's measurement tool was calibrated prior to photography each day using a calibration slide. Three observers independently measured an arbitrary "unbiased" group of 15 or more oocytes from each photomicrograph. Measurements were taken using the measurement tool in the opensource program ImageJ 1.53e for Windows that was precalibrated with the measurements embedded in each photograph by the DinoCapture software. Measurements taken by all three observers were compiled and mean diameter and SD, minimum diameter (min Φ), and maximum diameter (max Φ) were determined for each female on each sample day.

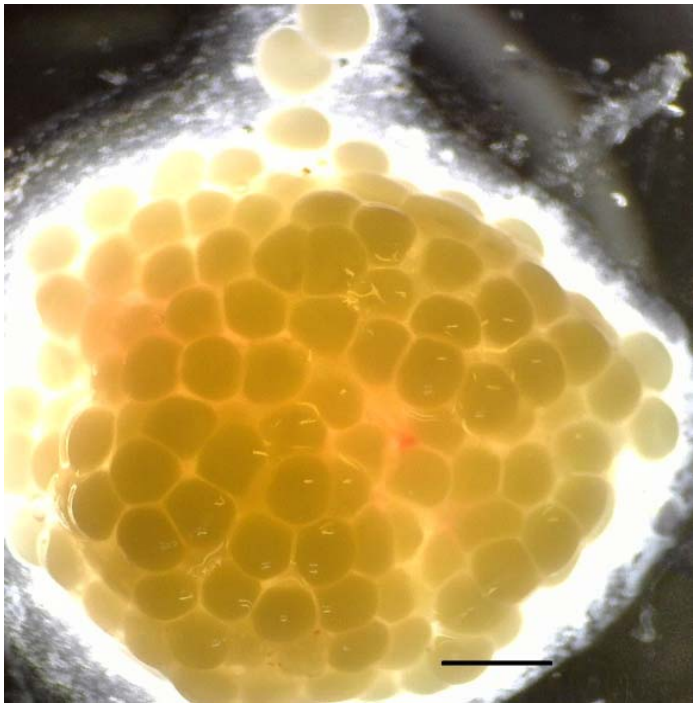


Figure 13. Photomicrograph of fresh mount of the gonadal biopsy of female #221989 on 1 October 2020 (scale bar = 1 mm).

Histological Analysis

Oocytes were examined and photographed using an OLYMPUS BX51 compound microscope with an OLYMPUS DP71 camera at magnifications of 4-10X (Figure 14). A subset of oocytes (~30) from each female were counted and classified into one of seven developmental stages: primary growth (PG), cortical alveolus (CA), late vitellogenic (LV), late vitellogenic with germinal vesical migration (GVM), late vitellogenic with germinal vesical breakdown (GVBD), POF (postovulation follicles) and hydration. The diameters (µm) of oocytes classified as LV, GVM, GVBD, POF, or hydration were measured by bisecting through the center of the cell using a calibrated ruler within the image processing software cellSens Standard v1.11 (OLYMPUS). In total, 2951 oocytes were categorized, and 1177 diameters were measured. Oocyte diameter averages and standard deviations for fresh and histological samples were calculated for each individual and for each treatment group and the control group. The proportional occurrence of each developmental stage was calculated for each individual and for each treatment group and for the sham injected group.

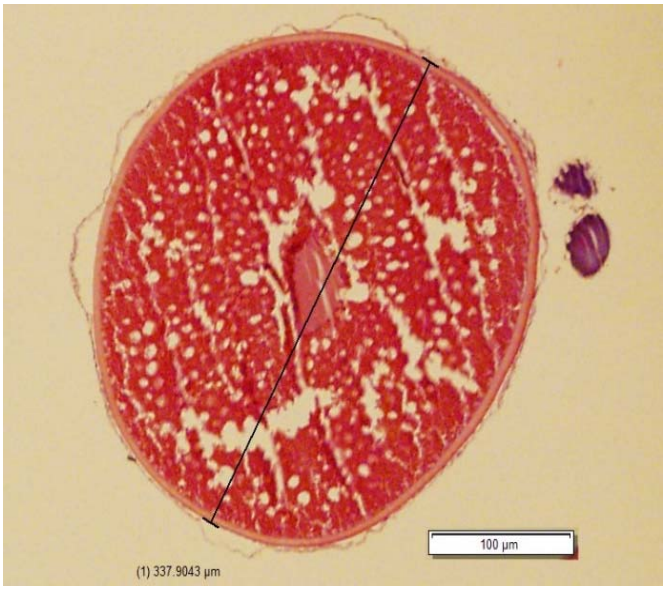


Figure 14. Photomicrograph of histological section from the gonadal biopsy of female #221989 on 1 October 2020 showing a late vitellogenic oocyte (scale bar = 100 µm).

Genetic Analysis

Pectoral fin clips were collected from all 32 broodstock individuals (males and females) and four replicates of mixed egg samples were taken from the egg collection tanks in the morning after each spawn. DNA was extracted, checked for quality and concentration, and sent

to CD Genomics (New York, New York, US) for 2B-RAD sequencing and quality filtering of low-quality sequences. 2B-RAD sequencing used a type IIB restriction enzyme (BsaXI) to fragment the DNA into small pieces making sequencing easier and more cost-effective while still providing a wide view of possible genetic markers.

Once received by HBOI, the post-quality filtering sequence data were processed with a series of computational pipelines with the genome of a closely related species, *Albula glossadona*, as a reference to find variable single nucleotide polymorphisms (SNPs; pronounced as "snips") as markers (Korneliussen et al., 2014; Miller-Crews et al., 2021; Rochette et al., 2019; Wang et al., 2012). All samples were from the same species, so they would generally have the same genome outside of some small percentage variations that can be used to discriminate individuals; in this case the analysis is looking at variations in the SNPs. These are single nucleotides within the same genomic sequence that are variable between individuals/samples. They can be used to estimate parentage by comparing the variations between DNA in the adult fin clip samples and DNA from the egg samples and then determining which adult has the most variations in common with each egg sample. These similarities in SNP variations were summarized as four Identity-by-State (IBS) matrices: Spawn 1 mixed eggs and females; Spawn 1 mixed eggs and males; Spawn 1 mixed eggs and females; and Spawn 2 mixed eggs and males. A larger IBS value associated with samples that had more variations in common.

These matrices were imported into RStudio (R Core Team, 2020; RStudio Team, 2020) and underwent hierarchical cluster analysis (HCA) using various R libraries (Galili, 2015; Suzuki & Shimodaira, 2006). In HCA, each sample is placed into its own cluster, then the two most closely related clusters/samples are combined, then the next two, etc. until all samples are combined into one cluster with many branches (a.k.a., a dendrogram). A dendrogram looks like a

"family tree" with each branch having two statistically determined p-values denoting the extent to which the branching is supported by the data. These p-values are on a scale of 0-100 with 100 meaning it is strongly supported by the data. For parentage analysis purposes, if a male/female branches with a mixed egg sample, then that means that male or female is most likely a parent. Miller-Crews et al. (2021) tested parentage analysis with mixed egg samples with known parents and found that a p-value cutoff of 80 was sufficient to indicate parentage.

Results: Spawning and Larval Rearing

On the mornings of October 5 and 6, 2020 eggs were noted in the egg collection tanks. Analysis of the video recordings indicated probable spawning activity, as indicated by much swimming activity and splashing at the surface followed by production of extensive foam on the water surface, at around 21:41 on October 4, 2020 and then again at 02:09 on October 6, 2020. A single frame capture just prior to a large surface splash at 20:40:59 on October 4, 2020 depicts two fish one following the other (Figure 15), in a pattern that is consistent with a male/female interaction seen in prior videos of bonefish spawning in aquaria at the Atlantis Resort in the Bahamas. Total spawn volume on October 5th was around 465 ml of

Figure 15. Screen capture from video depicting two fish in a typical spawning position with the female in lead followed by a male on October 4, 2020 at 20:40:49.

eggs, which is equivalent to approximately 260,000 eggs. The average number of eggs per milliliter was 557 eggs/ml. Fertilization rate was extremely high (> 90%). Fertilized eggs were then stocked into 8 kriesel tanks at a density of 15,000 eggs per kriesel tank. We decreased the stocking volume from the previous runs, as we were concerned that high density might affect larval survival. Eggs hatched around 24 hours after stocking. Water temperature was maintained at 25℃. Salinity was maintained at 30 ppt for half of the incubation tanks and at 35 ppt for the other half (total number of tanks was 8). The hatching success was similar when compared between the two salinities tested. Dissolved Oxygen levels were maintained at 130-150% saturation during the larval rearing. PH was maintained at 7.7-8.5 range. Water chemistry was maintained in favorable ranges (Alkalinity >100mg/L, TAN never got over 0.2 mg/L and Nitrite was never higher than 0.100mg/L).

We used a total of 10 kriesel tanks, all stocked at a density of 15,000 eggs per tank for both spawns. Overall, the quality of the first spawn was best and the second spawn was ultimately discarded after the poor hatch, so as not to pollute the entire rearing system. Yolk and oil droplet retention lasted 8-9 days, which resulted in first feeding beginning between days 8 and 9 post stocking. During that time, we observed a decrease in larval densities in the kriesel tanks. When first feeding started, only six kriesel tanks were left with leptocephalus larvae.

For this larval feeding trial, we used the omega 3 oil boosted hen's egg/krill emulsion diets that were made in-house to replicate the successful diet used by colleagues in Japan, who work with Japanese eel leptocephalus larvae. We cannot exactly replicate their diet, which uses the yolk of an endangered shark species which is not possible in the USA. The larvae were introduced to the emulsion feed at the beginning of exogenous feeding period (Day 9 post stocking or post hatch). The egg/krill emulsion diet was very oily. It formed a slick on the

surface and floated the whole time. To help with the water quality, feed was removed after 2 hours and a fresh ration or the egg based diet was added. We did not observe active feeding on this new emulsion diet as was observed in eels leptocephalus larvae. In addition, leptocephalus mortality continued to occur. By October 19th (Day 13 post hatch), all remaining larvae had starved. Nevertheless, this is the longest we were able to keep the bonefish leptocephalus larvae alive.

During the following full moon (October 29th, 2020), we did a final egg check on this group and found that most of the males had stopped producing milt. The female's egg size and quality were regressing (Figure 16).

Results: Fresh Egg Analysis

The pattern of egg diameter change over time for the oocytes measured from ovarian biopsies indicated that two of the fish had a pattern that suggested a high likelihood that they spawned, as indicated by a complete lack of sampleable ovarian tissue (i.e., oocytes so small or in low abundance that a biopsy sample wasn't achievable) and four had a pattern that suggested a moderate likelihood of spawning as indicated

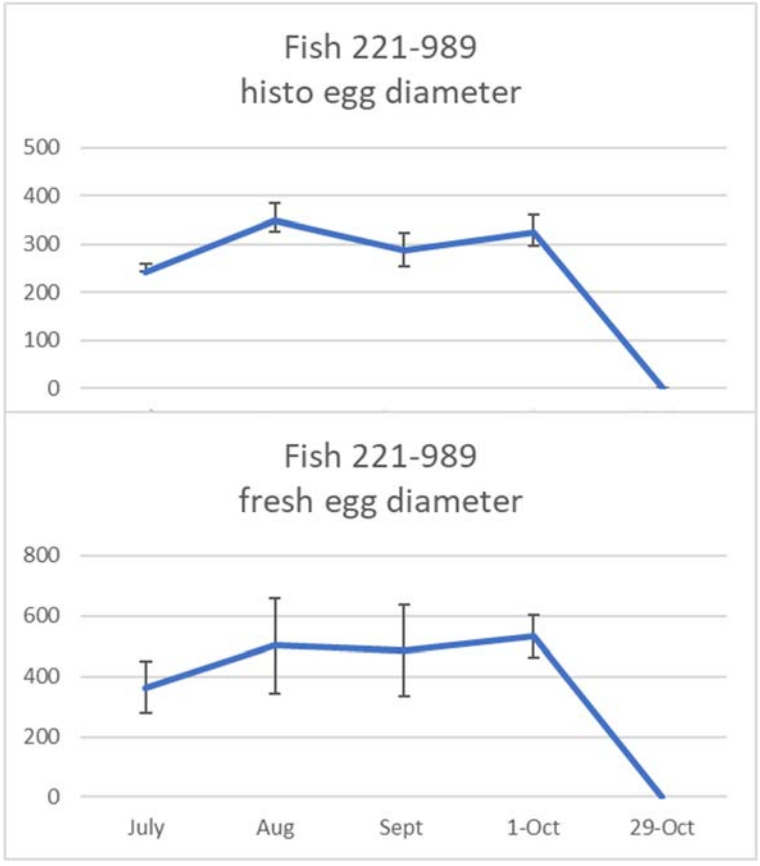


Figure 16. Fresh and histologically determined egg diameters for Fish Tag#221989 as an example, but which was ultimately determined to have spawned on October 5, 2020.

by a large decrease in oocyte diameter (Table 2). Eight others had a pattern that showed a decrease in oocyte diameter but not to the degree of the moderate or high likelihood groups.

Results: Histological Analysis

There was no significant difference in the average fresh oocyte diameter size between the different treatment groups and the sham group at any collection point. There was no significant difference in the average diameter of the histological oocytes between the sham group and treatment groups collected in July, August, and October 1st. In September, the sham and the 160 mg/kg SPE groups had significantly larger histological oocyte diameters on average than the 80 mg/kg SPE and 120 mg/kg SPE groups. The sham group had a larger average histological oocyte diameter on the October 29th sampling event.

At the July sampling, only oocytes classified as PG, CA, and LV were identified in the treatment groups and control group. In August, one oocyte was classified as GVBD in the 160 mg/kg SPE group. All other oocytes in all treatment and control groups were classified as PG, CA, and LV. In September, GVBD classified cells were identified in the control, 160 mg/kg SPE, and 80 mg/kg SPE groups. One POF classified cell was identified in the 160 mg/kg SPE group. Three cells were identified as being hydration in the 120 mg/kg SPE group. All other cells during the September sampling event were classified as PG, CA, and LV. For the October 1st sampling event, one oocyte from the 160 mg/kg SPE group was classified as GVM and five oocytes from the 120 mg/kg SPE group were classified as hydration. All other oocytes were identified as PG, CA, or LV.

During the sampling event that occurred three and a half weeks post-spawn, there was a decrease in oocytes categorized as LV or above across all experimental groups and the control with all cells categorized as PG, CA, or LV. Histological evidence suggests that a total 11

females had a high likelihood of being able to spawn during the two spawning events (Table 2). These 11 individuals had oocytes with a large histological diameter (≥300 μm) on the sampling event that occurred 3-4 days pre-spawn, but had no oocytes or oocytes categorized as PG or CA when sampled three and a half weeks after the spawning event. These eleven individuals included females from all experimental groups and from the control group (1 in the 160 SPE mg/kg group, 4 in the 120 mg/kg SPE group, 4 in the 80 mg/kg SPE group, and 2 in the control group). Parentage analysis will need to be completed to confirm these results.

Results: Genetic Analysis

The most likely statistically significant mother of all Spawn 1 replicates is PIT221989, which was dosed with SPE at 80 mg/Kg and implanted with a sGNRHa implant while the most likely statistically significant father was PIT180594, dosed with HCG at 1500 IU/Kg (Table 5). Every Spawn 2 replicate branched out, non-statistically significantly, with a different female. Replicate A was associated with PIT221944, dosed with SPE at 160 mg/Kg and implanted with sGNRHa, while replicate egg samples B, C, and D were associated with females PIT1019166, PIT221931, PIT221979, respectively, that were dosed with SPE at (160, 120, and 80 mg/Kg respectively) but not implanted with sGNRHa. All replicates were significantly associated with a single male, PIT221960, that was given a sham injection.

Table 5. Comparison of Parentage analysis methods indicating likely female and male (by PIT TAG Number) that contributed to spawn 1 on October 5, 2020, and Spawn 2 on October 6, 2020. Hormone treatment regimes for each female regardless of assessment method, and for each male identified by genetic analysis are indicated. Fresh egg minimum and maximum oocyte diameters (Φ) in µm are reported for the October 1, 2020 ovarian biopsy samples prior to the spawn dates.

		Hormone dosage(mg/kg) - Month 2020					Fresh Egg Diameter (µm)			Post-Spawn Parentage Assessment Method[2]		
Female ID	Hormone Treatment[1]	7-Jul	4-Aug	1-Sep	1-Oct	29-Oct	Oct minΦ	Oct maxΦ	Hormone Implant 1-Oct	Fresh Egg Φ Pattern	Histology Egg Φ Pattern	Maternal Genetic Analysis[3]
018-913	SPE	120	120	120	120	n/a	135.8	482.7	-	NL	NL	-
018-915	SPE	160	160	160	160	n/a	303.9	763.6	75 µg sGNRHa	ML	HL	-
018-957	SPE	160	160	160	160	n/a	45.9	390.6	-	NL	NL	-
019-166	SPE	160	160	160	160	n/a	151.3	655.1	-	LL	LL	2
180-582	SPE	80	80	80	80	n/a	197.3	727.0	75 µg sGNRHa	ML	HL	-
221-813	SPE	80	80	80	80	n/a	205.1	692.6	75 µg sGNRHa	ML	HL	-
221-825	SHAM	n/a	n/a	n/a	n/a	n/a	196.8	679.2	-	LL	HL	-
221-836	SPE	120	120	120	120	n/a	214.9	556.3	-	NL	NL	-
221-837	SHAM	n/a	n/a	n/a	n/a	n/a	176.8	629.8	-	LL	HL	-
221-878	SPE	120	120	120	120	n/a	263.8	643.9	75 µg sGNRHa	LL	HL	-
221-901	SHAM	n/a	n/a	n/a	n/a	n/a	96.0	262.6	-	NL	NL	-
221-913	SPE	120	120	120	120	n/a	282.5	679.3	-	LL	HL	-
221-916	SPE	160	160	160	160	n/a	191.6	724.8	-	LL	LL	-
221-920	SPE	160	160	160	160	n/a	151.1	368.8	-	NL	NL	-
221-931	SPE	120	120	120	120	n/a	200.6	769.1	-	LL	HL	2[4]
221-937	SPE	120	120	120	120	n/a	425.9	725.3	-	ML	HL	-
221-944	SPE	160	160	160	160	n/a	207.8	770.2	75 µg sGNRHa	NL	NL	2
221-959	SPE	80	80	80	80	n/a	349.2	671.7	-	LL	HL	-
221-979	SPE	80	80	80	80	n/a	200.3	679.2	-	NL	NL	2
221-981	SPE	120	120	120	120	n/a	347.6	676.8	-	NL	NL	-
221-986	SPE	160	160	160	160	n/a	169.5	448.5	-	NL	NL	-
221-989	SPE	80	80	80	80	n/a	330.4	728.9	75 µg sGNRHa	HL	HL	1
221-991	SPE	80	80	80	80	n/a	311.2	744.0	75 µg sGNRHa	HL	HL	-

1/ SPE=Salmon Pituitary Extract; SHAM=Bacteriostatic Water

2/ assessment classification HL = High likelihood; ML=Moderate likelyhood; LL=Low likelehood; NL=No Likelyhood

3/ Spawn Number indicated (1 = 10/5/2020, or 2 = 10/6/2020); Spawn1 - 1♂ male PIT Tag# 22180594 HCG; Spawn 2 - 1♂ male PIT Tag# 221960 SHAM

4/ Most likely Spawner based on Egg Φ Pattern Assessment

Captive spawn- February 2022

Just prior to the end of the captive spawning program in February 2022, as fish were being prepared for shipment to several public aquaria, *A. goreensis* spawned, and eggs were discovered the following day in the systems particulate filters. Unfortunately, no egg collector was set up to collect eggs as the task was deemed complete for the project. Therefore, the eggs did not survive and develop, likely due to mechanical damage in the filters. However, a total of 2.5 L of intact yet addled eggs were recovered. In 1 ml, we counted a mean of 739 (SD=84) eggs, for a minimum total of approximately 1,847,500 eggs recovered. This is a much larger number of eggs than in the prior two spawns of October 2020. Analysis of the security videos after the fact suggested multiple bouts of spawning throughout the night and early morning as indicated by increased activity, splashes at the surface, fish following each other (Figure 16), and subsequent production of foam on the water's surface in the tank.

Figure 16. Screen Captures of two separate putative spawning events each a few minutes apart from one another. Note the close juxtaposition of the two fish which is typical spawning behavior in each circumstance.

Natural Photo-Thermal Cycled Captive Broodstock Volitional Spawning Trials

In 2017 a large capacity conditioning/holding system was brought online designed to condition bonefish for spawning using natural light cycles and temperature cycles. This system was comprised of a 20,000 gallon, 25 foot diameter x 6 foot deep tank. This system was a closed recirculating aquaculture system equipped with a 3,000 gallon moving bed bioreactor, a 60 µm micro-screen drum filter for large solids filtration, a protein fractionator for fine solids filtration, and an ultraviolet filter that delivered at least 30,000 µW*sec/cm^2 of 256 nm UVC irradiation for disinfection. Multiple air lift driven egg collectors equipped with surface skimmers were installed around the perimeter of the tank to collect floating fertile eggs if a volitional in-tank spawn occurred.

Water temperature was controlled to some degree by a side looped heat pump heating/cooling system, but temperature generally matched outside environmental temperature. The chiller on the system kept the temperature from rising too high during the summer months (29-30 °C) or falling too low during the winter (18-19 °C). Light cycles were maintained with a timer controlled full spectrum LED light bulb system (dimmer capabilities to ramp up and ramp down light intensity over a chosen time period) similar to that described previously. Conditioning and spawning photoperiod cycle and thermal cycles in this system remained on the natural ambient cycles for the Fort Pierce, FL area. Full moon phases were also replicated by a single LED bulb set to a dim blueish color similar to moonlight.

Broodfish in this system were fed three times a day to satiation using the formulated gel-type broodfish diet we modified for bonefish. To determine if environmental cues alone are sufficient to induce spawning, there was no hormone manipulation done on this population of fish. We checked the egg collectors daily for any natural spawning during the three spawning seasons

(Fall, Winter and Spring of 2018, 2019, and 2020). The *A. vulpes* and *A. goreensis* broodstock in the tank were easily identified from each other since all fish in our breeding groups were individual tagged with microchip passive integrated transponder (PIT) tags that have unique identification numbers. No spawns were observed in this group during any of the natural spawning seasons. The *A. goreensis* were removed from the tank and the remaining group of *A. vulpes* was subsequently switched to hormone induction trials for the remainder of the project in 2021.

Embryological and Early Larval Development of Bonefish

The spawning event at Cape Eleuthera Institute provided the first embryos and larvae ever produced or studied in captivity. Subsequent spawns have added to the duration of development that could be characterized. In general, embryonic development was very rapid, with hatching occurring after 25-26 hours of development (Figure 17). Embryos hatched into morphologically primitive larvae (Figure 18), with a mean Notochord Length (= body length) of 3.36 mm. No mouth or digestive system was present, and eyes were just beginning to develop. The remnants

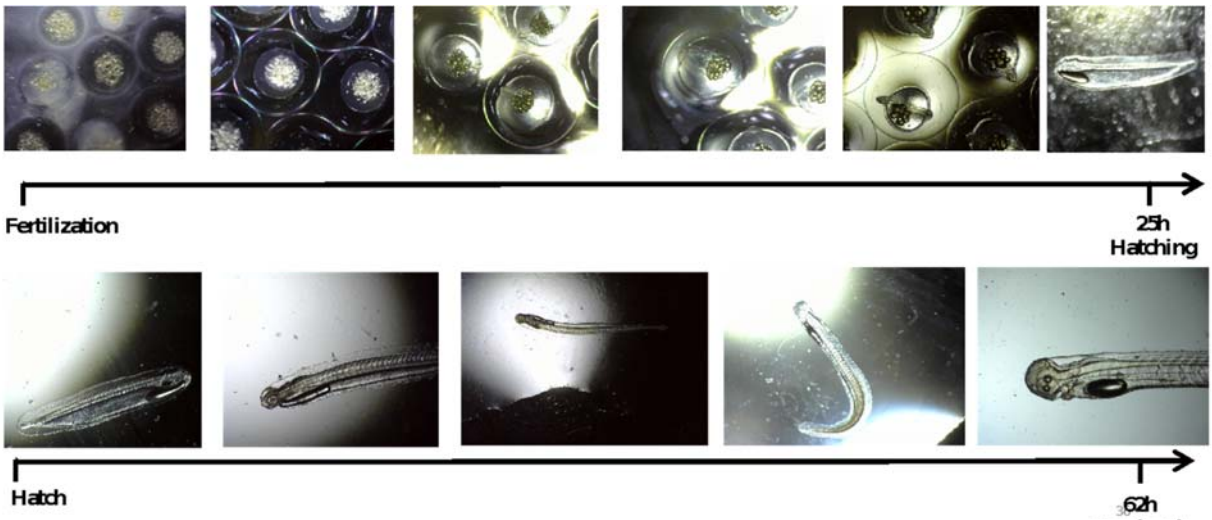


Figure 17. Pictures of bonefish eggs from fertilization to hatching (top) and leptocephalus larvae from hatch until 62h post hatch (bottom).

of the yolk extended nearly the full length of each larva, with oil droplets having coalesced into a single elongated droplet at the anterior of the yolk (Figure 24). Larvae grew at an average rate of 1.7 mm NL per day to a mean length of 7.4 mm NL at 52 hours post hatch (hph). Larvae nearly completed full utilization of their yolk reserves by 56 hph, when the final larvae died. Larvae initially oriented vertically in rearing containers, with heads oriented towards the surface and generally remained motionless, although occasional sporadic darting movements were observed.

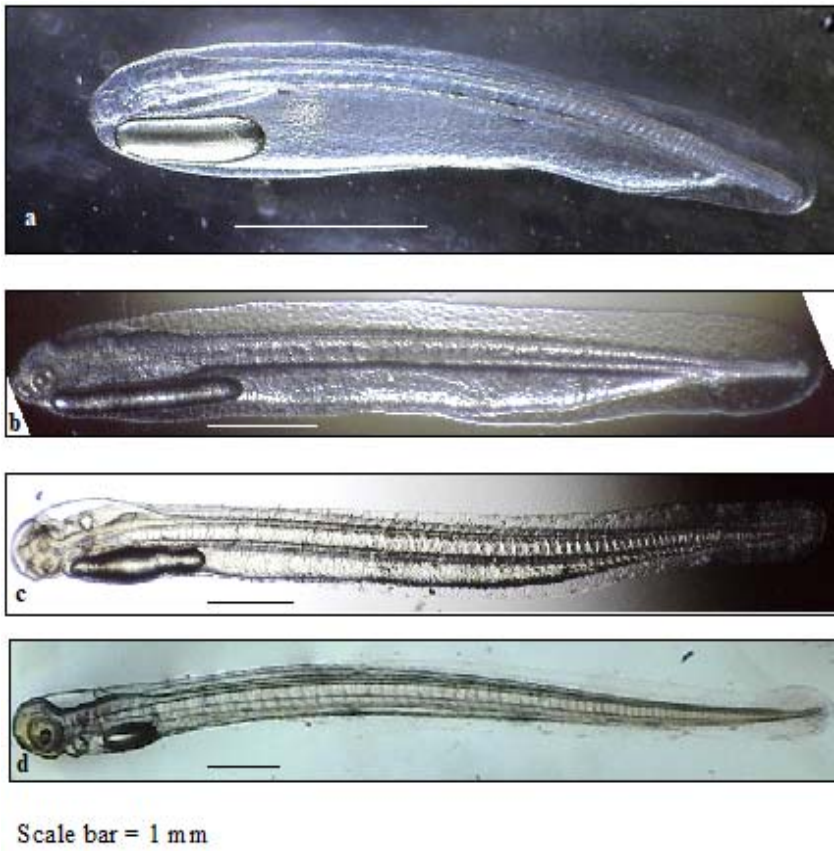


Figure 18. Microscopic examination of larvae of A. vulpes. a) Larvae at 2 hours post hatch (hph). b) Larvae at 10 hph. c) Larvae at 30 hph. d) Larvae at 52 hph.

Figure 19. Kreisel tanks used to rear Japanese leptocephalus larvae

In subsequent larval rearing efforts aboard the M/Y *Albula* and at HBOI, we reared the larvae in Kreisel tanks (Figure 19), which is a rearing technique used by Japanese researchers to rear leptocephalus larvae of the Japanese Eel, *Anguilla japonica*. Thanks to a collaboration with Dr. Katsumi Tsukamoto at Nihon University, Fujisawa, Japan, our graduate student, William Halstead, traveled to Japan to learn their techniques and used this knowledge to construct the specialized system for the Kreisel tanks at the aquaculture facility at HBOI. Eggs from broodfish that were spawned at Great Abaco, Bahamas, during the November 2019 cruise of the M/Y Albula were transported back to HBOI and the larvae were reared through eight days post hatch (Figure 20). These larvae developed a complex jaw with long sharp teeth and fully pigmented eyes.

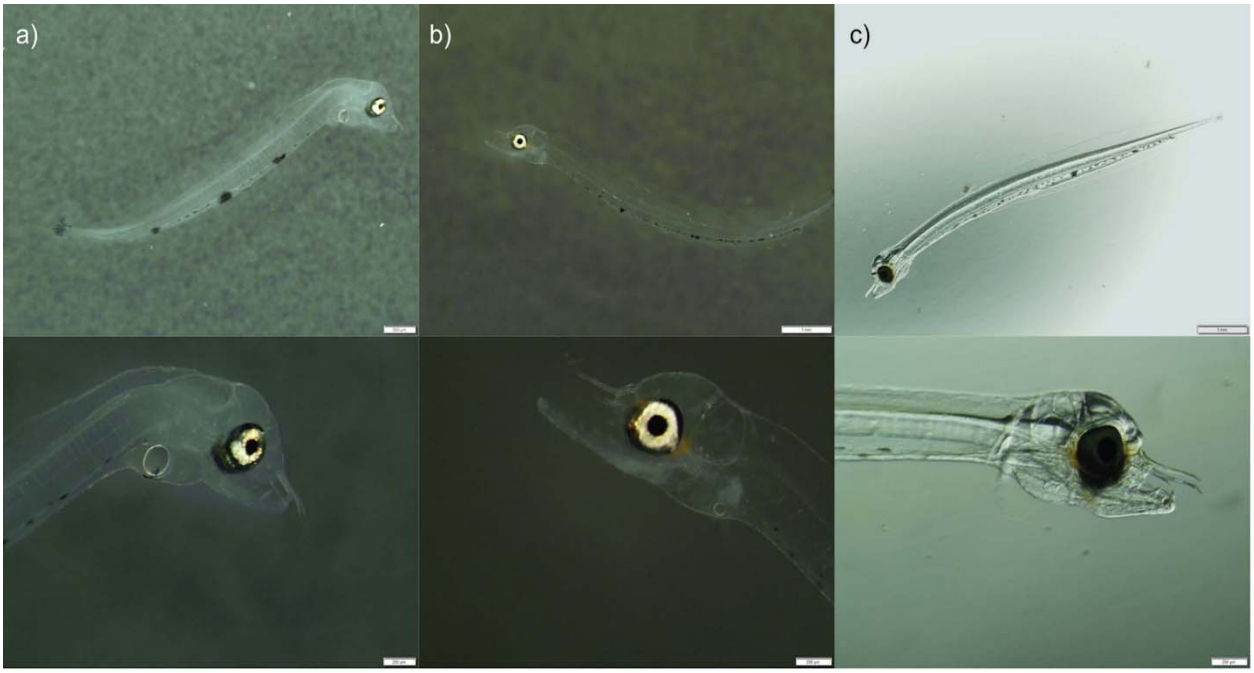


Figure 20. Micrographs taken at varying stages of *A. vulpes* larval development. Top: full photographs of larvae; bottom: close-up of head region. From left to right: a) 75 hph, b)120 hph (start of exogenous feeding), and c) 192 hph. Photos show a steady a steady decrease in oil droplet (at arrow) size after 75 hph. Broodfish from Great Abaco, Bahamas spawned November 2019 with larvae reared at HBOI.

The larvae from the broodfish at HBOI that were captive spawned in October 2020 were reared up to 11-12 days (October 5 and 6, 2020 until October 17, 2020). These are the longest-lived larvae reared (Figure 21). They were noted to be picking at "bacterial" flock on the Kreisel tank's surface but did not appear take the emulsion diets offered. Development of the *A. goreensis* was similar to *A. vulpes* but slightly delayed with teeth developing a day or so later.

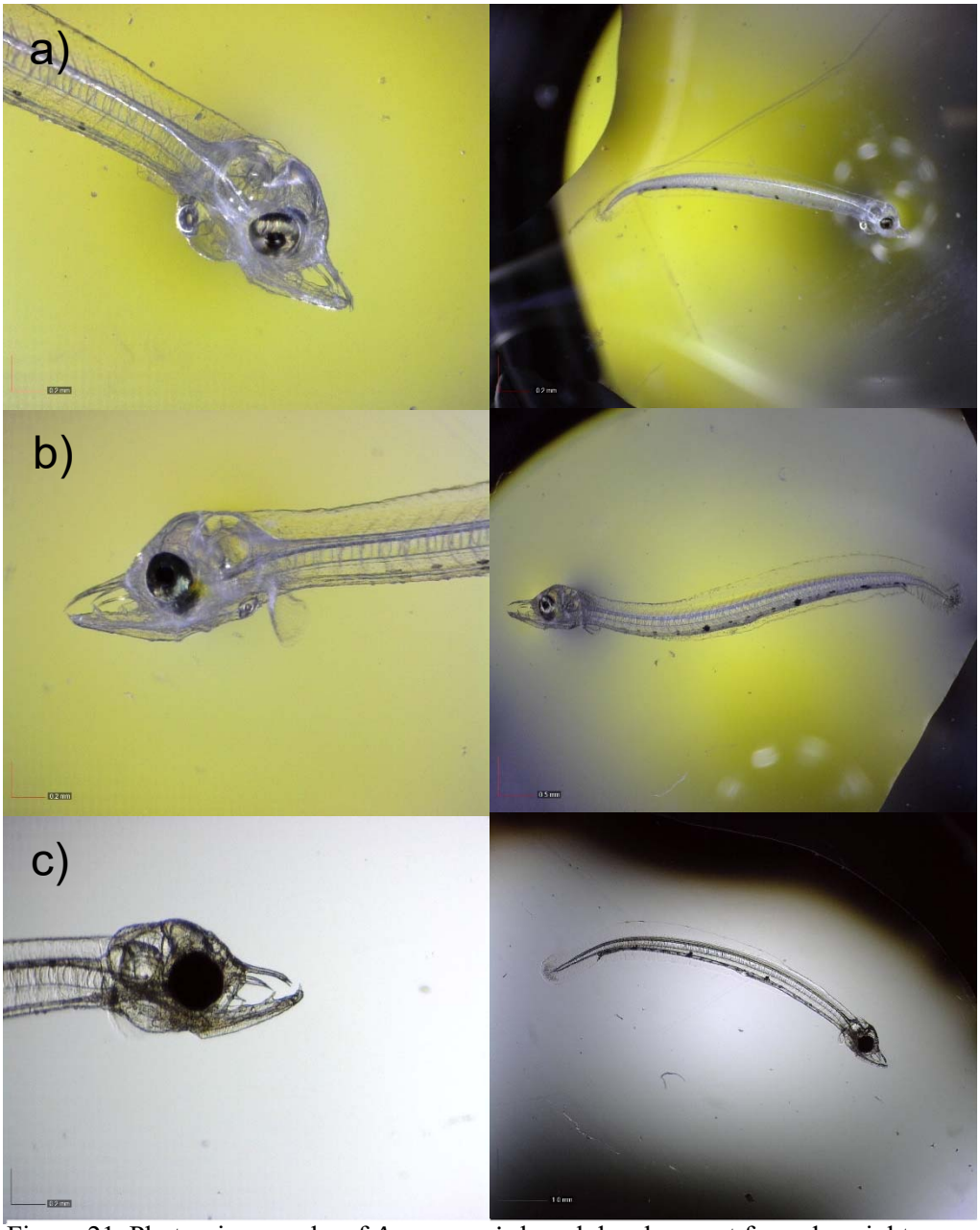


Figure 21. Photomicrographs of *A. goreensis* larval development from day eight through 12. From top to bottom: a) day seven, b) day eight, and c) day 12. Left Panels show detail of head and right panels show whole body.

Husbandry of Wild-Caught Late Leptocephalus Larvae

Leptocephali can be collected from the wild using light traps. These cylindrical nets have a light suspended inside that draws the larvae inside and the net design prevents escape. Several of these light traps were deployed in the Indian River Lagoon at sites where late stage leptocephalus larvae might occur (e.g. inlets, bays, offshore). Depending on the time of year, leptocephali from bonefish (*A. goreensis*), tarpon, or ladyfish may be caught, as well as some eels.

Thirty leptocephali were collected, nearly all in the Harbor Branch Channel. The larvae that were sorted from the light traps were kept in 6 L jars with light aeration. Larvae were monitored daily and most mortality was observed shortly after capture. Water quality was maintained by full, daily water exchanges, which is also when any mortalities are removed.

The leptocephali were identified as ladyfish based on fin placement. As these were caught inshore, they were in the settlement stage and not feeding. After one week, brine shrimp *Artemia salina* were fed to the larvae to test their appetite and active feeding was observed and verified by the presence of *Artemia* in their gut.

After another week, once the larvae were actively feeding on *Artemia*, they were moved to a larger 20 L tank set up for water flow-through. Once the larvae were accustomed to their new tank and feeding successfully again on *Artemia*, we began weaning them onto a dry prepared-pelleted diets (Otohime), which are more nutritious than *Artemia*. Pellets were readily ingested after 48 hours and we ceased feeding *Artemia*. One week after the switch to pellets, a larger pellet was tested. Increasing pellet size as the larvae grow is important for optimal growth and the larvae switched to the larger pellet successfully.

Since these were ladyfish and not bonefish, we were mainly focused on navigating the challenges of first feeding, weaning off of live feed, and changing diets after metamorphosis from the leptocephalus stage into juveniles. These critical checkpoints in larval development are typically when high mortality can occur in many marine fish. Based on the success of transferring ladyfish through these checkpoints, the chances of successfully repeating this with bonefish is high. Having served their purpose for the project, the juvenile ladyfish were donated to the aquarium at the HBOI Ocean Discovery Visitor Center, where the staff educates the public about the Bonefish Research Restoration Program and other projects at Harbor Branch.

Assessing physical qualities and fatty acid profiles of early-stage bonefish (*Albula goreensis*) leptocephalus larvae reared in captivity

Larvae from previously described captive spawning events were used to determine physiological characteristics. A sample of larvae was collected daily for up to 3 days post-hatch and biometric data was recorded. To assess their nutritional content, which is derived from the lipids and proteins packaged into the eggs, total fatty acids (polar and neutral) were extracted from egg and larvae samples and analyzed using GC-MS detection methods. Larvae samples showed a general decrease in polyunsaturated fatty acids (PUFAs) coinciding with growth after hatching, supporting the notion that PUFAs are utilized for growth and metabolic processes.

Fatty acid profiles (Table 6) showed an overall increase in PUFAs over time and a decrease in saturated fatty acids (SFAs) across all samples, in terms of relative percentage of wet weight (%/WW). All samples showed the highest abundance in PUFAs across all stages which made up about 50% of total FA content in samples (Table 6). Both egg and larvae samples were most abundant in DHA, EPA, and C16:0. Relative percentages of ARA were lower in egg samples than in larvae samples. All samples showed high relative percentages of C14:0, C18:0,

C16:1, and C18:3 n-3 (alpha linolenic acid or αLNA). Larvae showed lower relative percentages of C16:0 compared to egg samples. Non-metric multidimensional scaling (NMDS) results showed grouping between eggs indicating similarity in content of the fatty acids (Figure 22B). Fatty acid profiles changed over time with most of the EFAs including DHA and EPA decreasing by the last day of sampling at about ~90 hpf. Egg sample FA profiles were different from larval samples and contained a higher relative percentage of EFAs such as DHA and EPA. Early-stage larvae (30-40 hpf) showed similar profiles to each other but were different from those of later stage larvae (55-65 hpf).

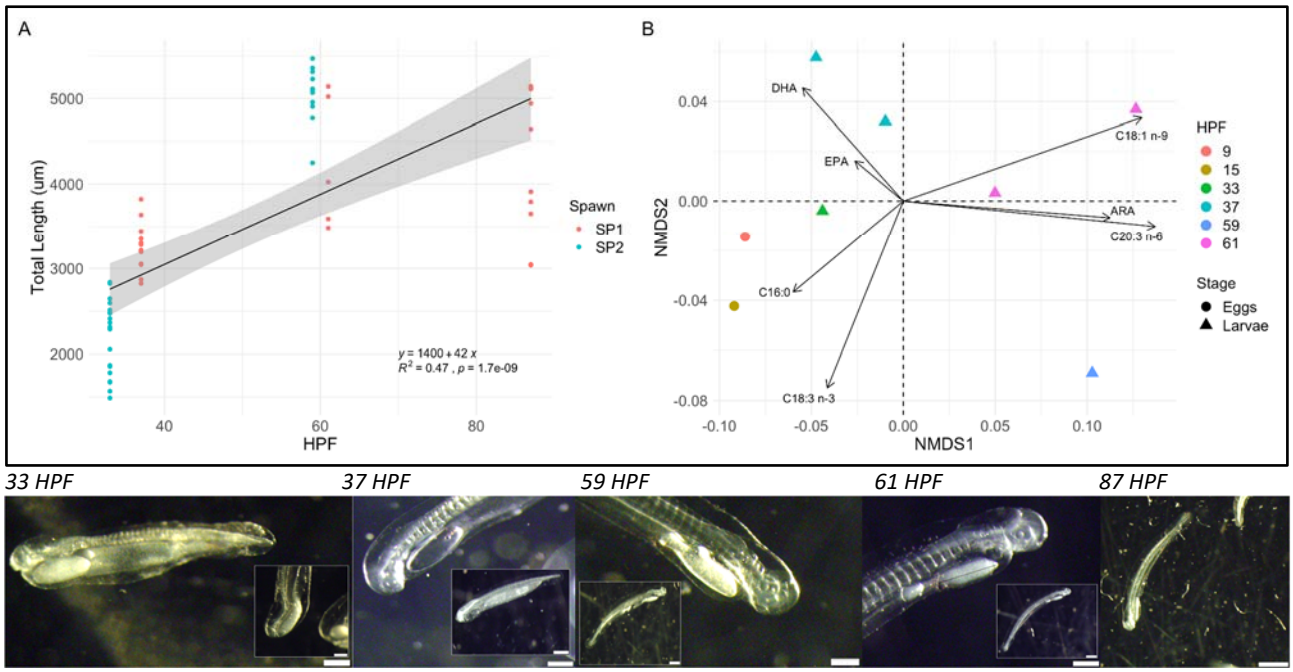


Figure 22. (A) Trend analysis using linear regression showing positive change in total length over time (in um) in relation to hours post fertilization (HPF) from 30-90 HPF. Spawn 1 is shown with orange dots and spawn 2 is shown with teal dots (B) Non-metric multidimensional scaling (NMDS) of based on relative percentages showing fatty acid associations with sampling times in hours post fertilization (HPF) and sample stages (eggs vs. larvae) Circle points show egg profiles while triangle points show larval profiles. (C) Photos of larvae from 33 87 HPF. Oil droplet shows visual depletion from about 30-60 HPF. Cephalization and myomere development are apparent in 37 HPF samples (~13 hours post hatch). Elongation and mouth development are apparent at about 59 HPF (~35 hours post hatch).

Table 6. Fatty acid profiles by stage (hours post fertilization or HPF) shown in terms of relative percentage (%/DW or WW) of total lipids. Sample groups with n>1 are expressed as mean ± SD. Key: HPH – hours post hatch, SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, ARA – arachidonic acid, EPA – eicosapentaenoic acid, DHA – docohexaenoic acid

STAGE	**Eggs (%/DW)**		**Larvae (%/WW)**			
HPF	**9**	**15**	**33**	**37**	**59**	**61**
C14:0	5.35	5.83	7.18	6.84 ± 0.25	7.38	7.27 ± 0.04
C16:0	17.59	17.29	14.52	12.17 ± 1.21	12.63	12.54 ± 1.86
C18:0	5.20	4.65	4.28	3.91 ± 0.72	3.32	2.39 ± 0.50
C20:0	1.69	1.78	3.65	3.47 ± 0.12	0.00	0.00 ± 0
C24:0	0.00	0.00	0.00	0.00 ± 0	0.00	0.00 ± 0
ΣSFA	**32.69**	**32.37**	**32.46**	**29.12**	**25.18**	**23.92**
C15:1	0.00	0.00	0.00	0.00 ± 0	0.00	0.00 ± 0
C16:1	8.37	8.13	7.06	8.07 ± 1.12	5.87	9.90 ± 0.19
C18:1	3.89	5.74	4.00	3.04 ± 0.88	5.23	4.64 ± 2.96
C18:1 n-9	3.04	2.36	2.93	4.02 ± 0.47	6.04	6.19 ± 2.83
C20:1	0.63	0.72	0.99	0.54 ± 0.21	0.00	0.00 ± 0
ΣMUFA	**20.19**	**21.46**	**20.90**	**17.03**	**25.06**	**20.92**
C18:2	3.05	3.19	3.98	4.23 ± 0.02	7.05	6.25 ± 0.54
C18:3 n-6	0.00	0.00	0.00	0.00 ± 0	0.00	0.00 ± 0
C18:3 n-3	5.34	5.42	5.47	4.80 ± 0.86	7.59	3.48 ± 0.73
C20:2	1.75	1.94	0.16	3.58 ± 0.02	0.16	7.46 ± 0.62
ARA	2.82	2.65	3.61	3.62 ± 0.25	6.43	5.97 ± 0.38
EPA	10.43	9.70	10.44	10.65 ± 0.25	9.48	9.58 ± 0.64
DHA	22.67	22.15	21.33	25.40 ± 1.54	15.92	19.55 ± 0.70
ΣPUFA	**47.12**	**46.17**	**46.63**	**53.86**	**49.76**	**55.16**

Juvenile Bonefish Husbandry

Juvenile bonefish were collected at numerous locations within the Indian River Lagoon. Captured juveniles were transported back to the HBOI Aquaculture Park, where they were placed into quarantine.

While in quarantine, the fish readily adapted to feeding on to frozen and thawed mysis shrimp as a diet. The fish preferred feeding on the bottom of the tank, so a pipe was installed to direct the feed being offered to the tank bottom. Juveniles were weaned from mysids and onto commercially prepared pellets and ultimately grown to adulthood.

Juvenile Bonefish Habitat

Identification of juvenile bonefish nurseries in South Florida remains as one of the largest unanswered questions in the Florida bonefish life cycle. The search for bonefish nursery habitats in South Florida spanned 2019 through 2021, with four-day sampling periods January through June. However, efforts ceased March 2020 through January 2021 due to COVID-19. Two gears were used to search for nursery habitats: a 21.3 m center-bag seine with a 3.1 mm stretch mesh, a baited/baitless remote underwater video (BRUV/RUV) designed to sample shallow intertidal waters as well as deeper waters up to 5 m.

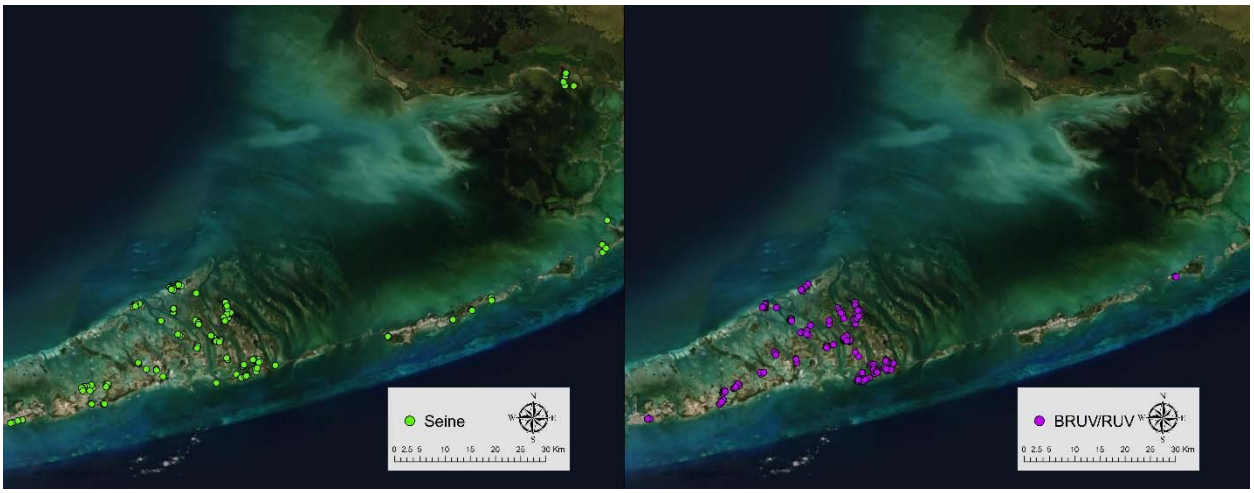


Figure 23. Haphazard seine and BRUV/RUV deployments for efforts spanning January through June of 2019, 2020, and 2021. No effort was put forth during the COVID-19 pandemic (March 2020 – January 2021.

In total, 170 seine deployments, 144 BRUV and 31 RUV deployments (approx. 15750 min) were conducted in a haphazard manner, with basins and sites selected based upon the ability to sample given local conditions (Figure 23).

Sampling focused mainly on the lower Keys, with opportunistic middle Keys and Everglades National Park sampling as well. Juvenile bonefish were only caught/observed using the seine gear (Figure 24).

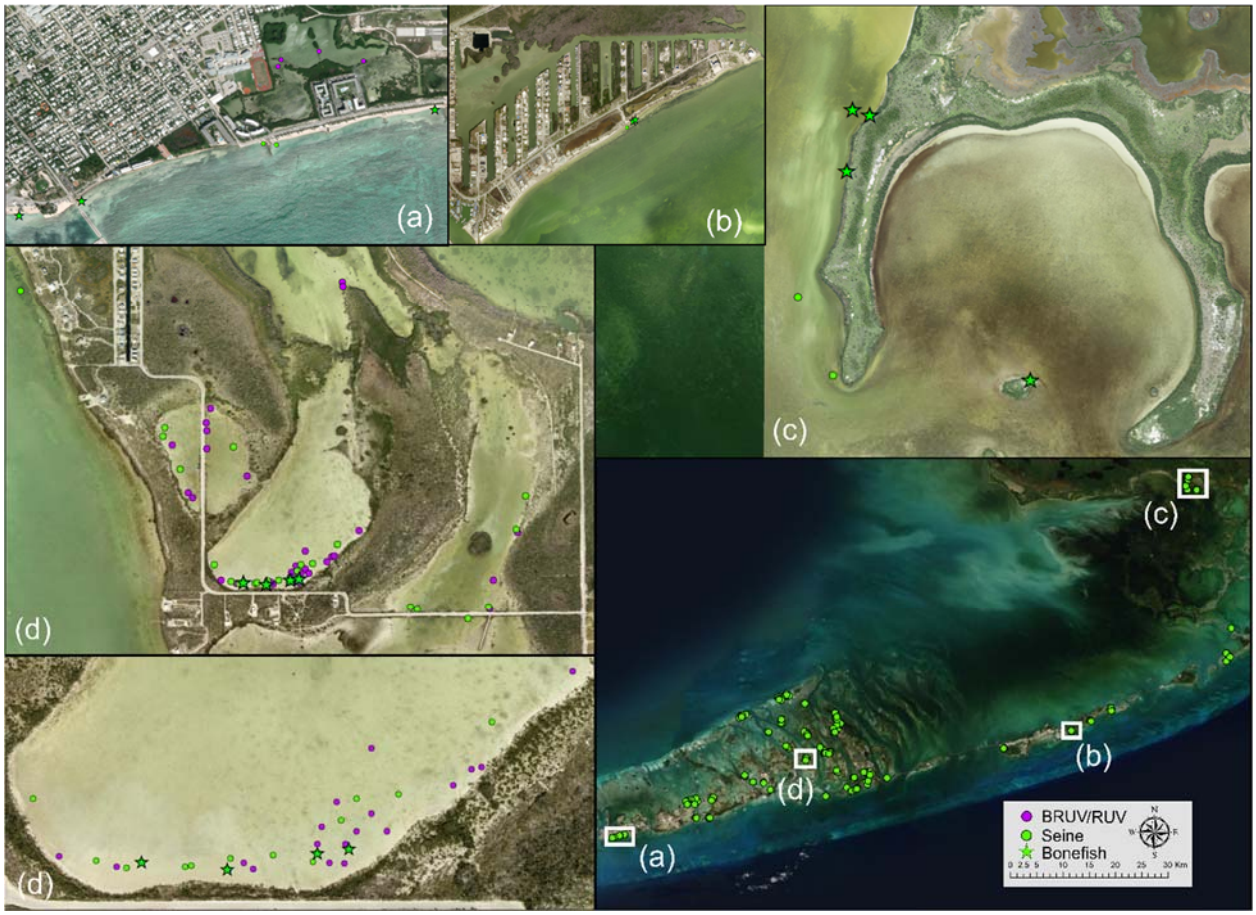


Figure 24. Seine and BRUV/RUV sets in areas where juvenile bonefish were caught. Juvenile bonefish presences are noted with stars. *Albula vulpes* were only caught within Garfield and Rankin Bights (c) and the embayment on Big Torch Key (d).

Bonefish were caught along the windward sandy beaches of Key West (n=34) and Cocoa Plum Beach Marathon (n=3), and within the shallow protected embayments of Rankin Bight (n=2) and Garfield Bight (n=12) of Everglades National Park (ENP) and a north-facing embayment within Big Torch Key (n=7). Bonefish caught on Key West and Marathon were all identified as *A. goreensis* with six unknowns. Bonefish caught within Rankin Bight were *A. vulpes*, and within Garfield Bight a co-occurring mix of two *A. vulpes*, three *A. goreensis*, five *cf A. vulpes*, and two unknowns were caught. All but one unknown fish were identified as *A. vulpes* within the Big Torch Key Embayment. *A. vulpes* were caught in February 2021 within ENP and April and May 2021 within the Big Torch Key embayment. The catches of *A. vulpes* within the Big Torch Key

embayment is a novel finding, as collaborator Dr. Jennifer Rehage has previously documented instances of *A. vulpes* within Garfield Bight (May 2018) and nearby Terrapin Bay (May 2017).

While catches of *A. vulpes* juveniles were few despite significant effort, a framework from which to direct future sampling efforts was established based upon conditions within Garfield Bight, Rankin Bight, and the embayment within Big Torch Key. The following is an itemized conceptual framework of what comprises bonefish nursery habitat within South Florida:

Characteristics of Lower Keys Bonefish Nursery Habitat

1. Within proximity (<18 miles) of the Gulf of Mexico
2. Within proximity of strong inflow from the Gulf (examine sediment flow via satellite)
3. Embayment allowing for protection from east and west winds, as close to 360 degrees as possible
4. Inlet direction allows for unidirectional larval transport from the Gulf of Mexico
    a. Inlet benthic composition (vegetation) does not impede leptocephali transport and survival
5. Low tidal flux/energy
6. Depth < 0.8 m
7. Away from shoreline ~10-40 m
8. Shoreline composed of black mangrove (*Avicennia germinans*) pneumatophores and dwarf mangrove (small fringe mangroves)
9. Will not be found up against overhanging mangrove
10. Shoreline has gradual slope or a lip leading to the black mangrove (creates a basin-like shoreline interface)
11. Benthic composition
    a. Sediment: fine grain mud/sand/shell hash, with mud 0.1-0.6 m deep
        i. Oxic/anoxic sediments are not significant based on Lower Keys/ENP (oxic/anoxic)
        ii. Can be atop limestone, but no exposed limestone
    b. Vegetation: sparsely vegetated. Bare mud/sand does not yield bonefish. Most commonly found at a distance from shorelines with bare mud/sand composition and along the interface the bare substrate and sparse vegetation.
        i. *Halodule wrightii* – most commonly associated. Low density grass
        ii. *Syringodium filiforme* – just as important as *H. wrightii*. Low density grass
        iii. *Penicillus capitatus* – moderately abundant, but does not grow in dense patches
        iv. *Acetabularia* sp. – not abundant or dense
    c. ***Bad Vegetation
        i. *Thalassia testudinum* – too much structure and wide profile
        ii. *Laurencia* sp. – grows over *Thalassia* and has too much structure.
            1. BIGGEST DANGER TO HABITAT INTEGRITY
        iii. *Batophora* sp. – can grow densely and is usually found against shoreline
            1. Often habitat for sheepshead minnows *Cyprinodon variegatus*
12. Temperatures and salinities can be up to 33.5 °C and 44 ppt, respectively

    a. Loss of equilibrium study by M. Thurman and catches by FWC within the Indian River Lagoon shows tolerance down to 5 ppt, though differences may exist between *A. vulpes* and *A. goreensis,* the species most likely observed in the IRL.
13. The presence of ichthyofauna of a similar functional ecology, roving benthic invertivores, is indicative of potential nursery habitat
    a. Primarily Eucinostomus spp.
14. The presence of organisms with similar dispersal states during the larval phase may also inform sampling, i.e., overlap with current-driven dispersal from offshore to inshore
    a. Pink shrimp *Farfantepenaeus duorarum* and brown shrimp *Farfantepenaeus aztecus*

Tracking spawning movement and characterization of offshore movement

Data describing the offshore spawning movements of bonefish are limited, but of high importance due to the economic value and conservation needs of the species and genus throughout their distributions (Adams et al. 2014; Fedler 2013; Fedler 2018; Filous et al. 2019). Danylchuk et al. (2011) used both passive and active acoustic telemetry techniques to characterize the timing and location of offshore spawning movements in Cape Eleuthera, The Bahamas; the first study to do so. Logistical and technological limitations of both the passive and active acoustic equipment precluded any determination of spawning depth, but did reveal consistent offshore movement behaviors towards the shelf edge and waters exceeding 42 m, followed by a return to the shallow-water flats habitat. Danylchuk et al. (2019) provided a more detailed description of pre-spawning behavior and offshore movements from active acoustic tracking efforts from 2013 and 2014 in Andros and Abaco, The Bahamas. The 2013 Abaco active tracking event yielded the first ever recording of diving movements during the offshore spawning migration. However, the description of fish vertical movement through the water column was restricted due to the depth rating (50 m) of the pressure sensor within the acoustic tags. The objective of building upon the efforts and data of Danylchuk et al. (2019) to provide a complete description of the spawning movements, behavior, and spawning habitat was undertaken, with efforts put forth to locate PSAs and use active acoustic telemetry to track fish

offshore in Abaco, the Berry Islands, Long Island, Acklins, and Bimini for the 2018-2019 and 2019-2020 spawning seasons.

The most successful efforts took place on Abaco, which allowed for a longitudinal comparison of spawning behaviors in 2013, 2018, and 2019. In 2018 and 2019, Vemco continuous pressure and temperature tags (V9TP, 9 mm diameter, 31 mm long, 4.9 g in air, period 1000 ms) with a 250 m depth limit were surgically implanted (Innovasea Systems Inc., Massachusetts). Each tag emitted a unique frequency at an interval of 1000 ms with an assumed detection range of 300 m (Melnychuk and Christensen 2009). The maximum number of at large V9TP continuous acoustic tags was limited to 6 due to the number of available unique frequencies manufactured. The number of individuals tracked offshore from the PSA location were less than the number of tags deployed due to predation events, tag failures, and lost contact with tagged fish during tracking efforts. Initial offshore movements of the entire PSA school were visually confirmed, though the number of individuals successfully tracked through the initial dive ranged from one (2013 and 2019) to three (2018) individuals. PSA formation and movement offshore varied in relation to lunar and solar position across years, with PSAs forming between one and five nights prior to the full moon and offshore movements beginning between 2 minutes before sunset and 55 minutes after sunset. The direction of offshore movements and final geospatial location – noted as a spawning event in 2013 and 2019, and where the fish were lost in 2018 – also was variable (Figure 25).

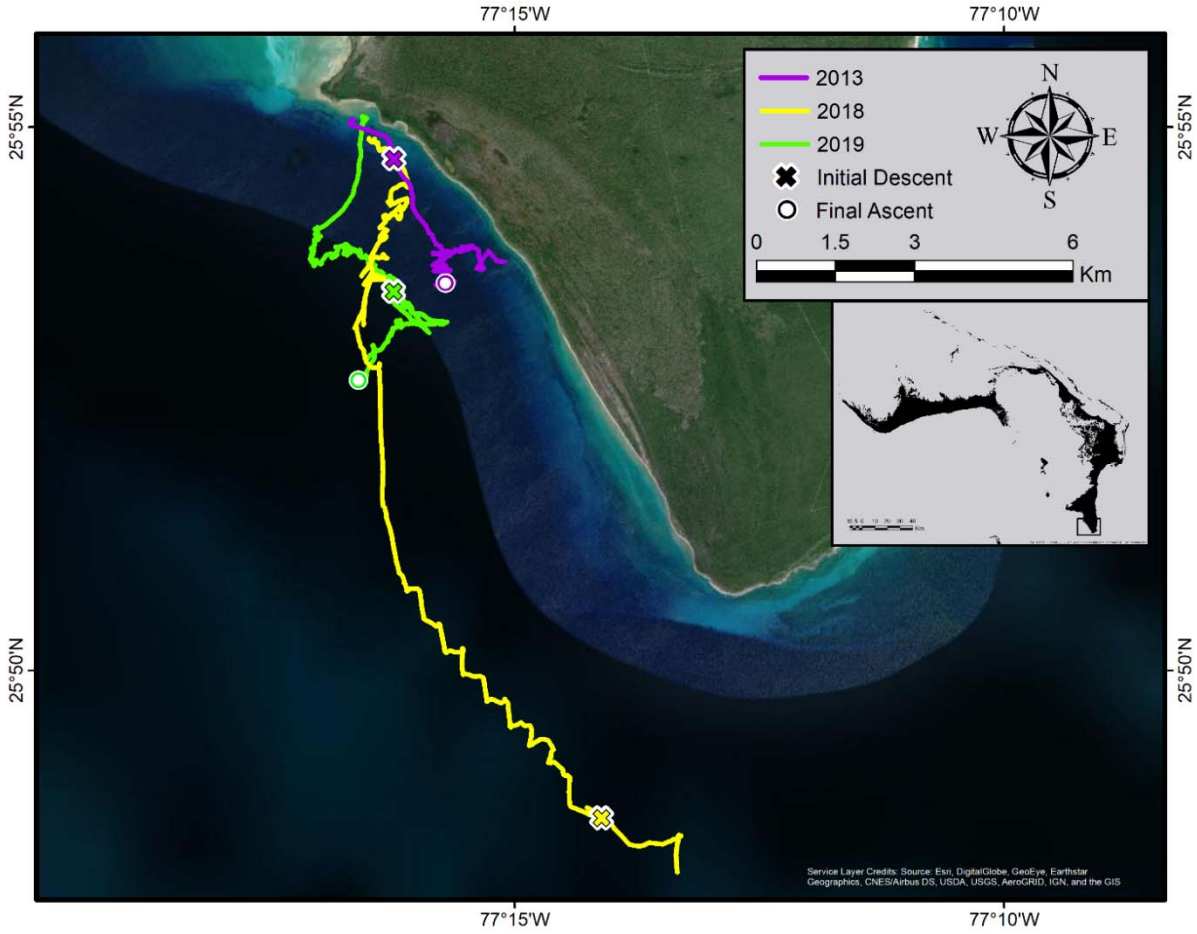


Figure 25. Spawning migration pathways of bonefish tagged at a PSA location in South Abaco, The Bahamas. Across all three years, an initial descent from surface movements was observed (indicated by X). In 2013 and 2019, the final ascent was observed (indicated by O).

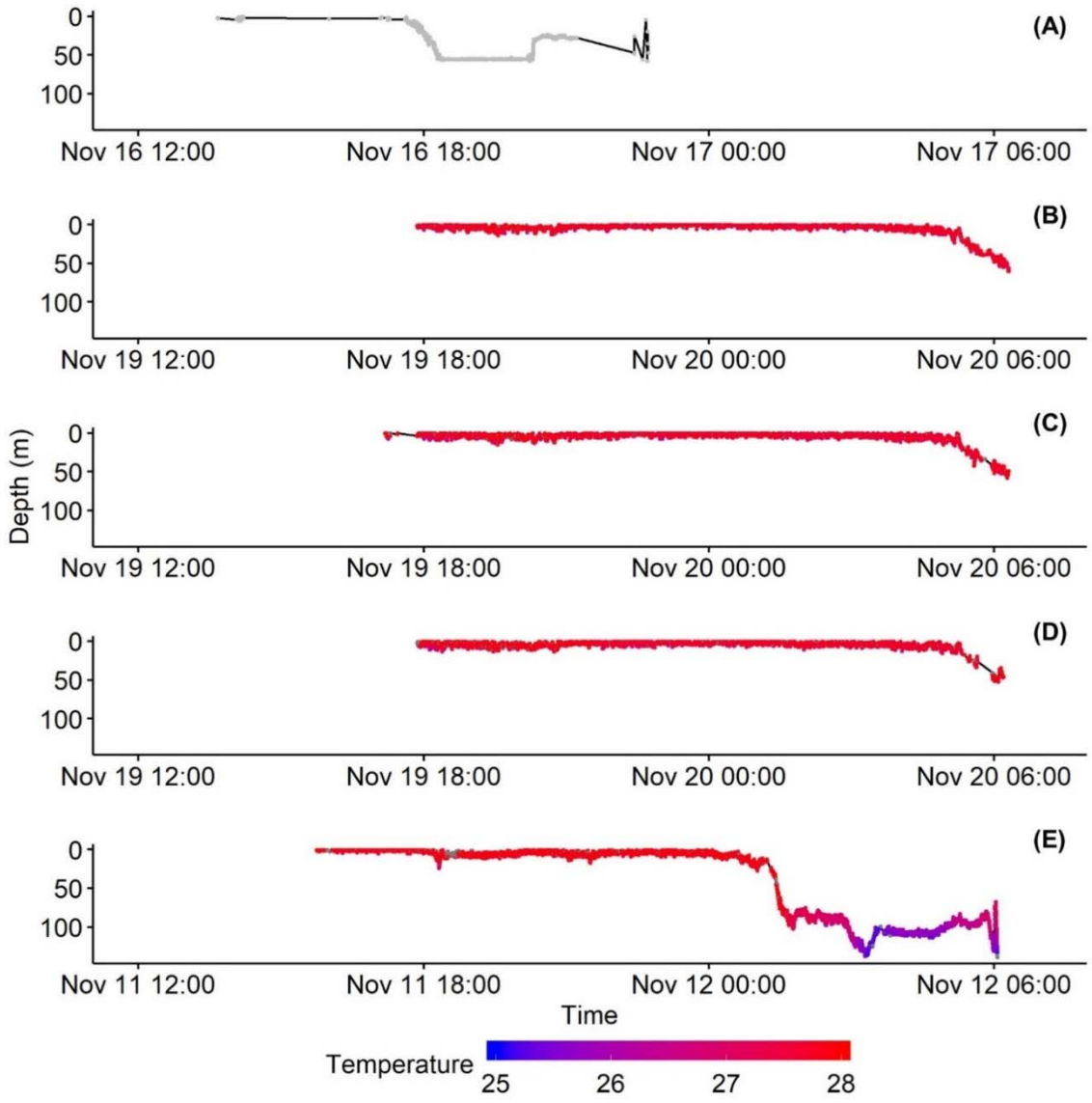


Figure 26. Dive profiles for actively tracked bonefish in 2013 (A) (Danylchuk et al. 2019), 2018 (B, C, D), and 2019 (E). Acoustic telemetry tags were depth limited to 50 m in 2013, and tags did not have a temperature sensor. Active tracking in 2018 ended when the fish were lost descending below 58 m. Bonefish were tracked in 2019 up until the tag was ejected in a likely spawning event.

Depth profiles in 2013 (see Danylchuk et al. 2019) and 2018 provided limited information due to equipment limitations and rough seas. However, in 2019, a complete spawning cycle was observed (Figure 26). During the offshore migration, the tagged fish was observed within the aggregation via sidescan sonar and CHIRP sonar, confirming that the fish was moving with the collective (Figure 27). The deepest depth reached by the fish during the track was down to 137.9

m, which is the deepest depth at which a bonefish has ever been recorded. The complete track in 2019 culminated with a rapid descent to 131.0 m immediately followed by a rapid ascent to 67.3 m, upon which the acoustic telemetry tag was expelled following oscillatory movements (Figure 28).

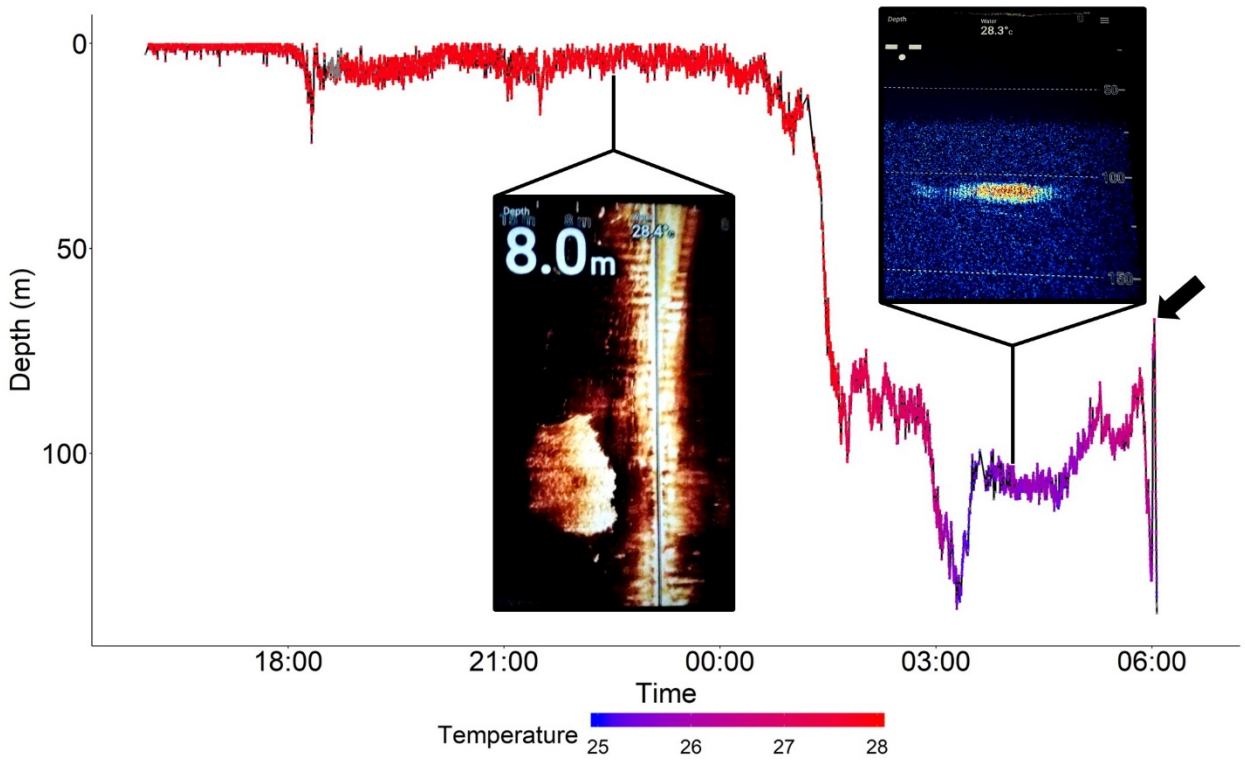


Figure 27. Dive profile of the female bonefish tracked during the 2019 spawning migration, colored to temperature transmitted by the acoustic telemetry tag. Sidescan and CHIRP sonar images of the aggregation are inset at times that the boat passed over the moving aggregation. Black arrow indicates tag ejection.

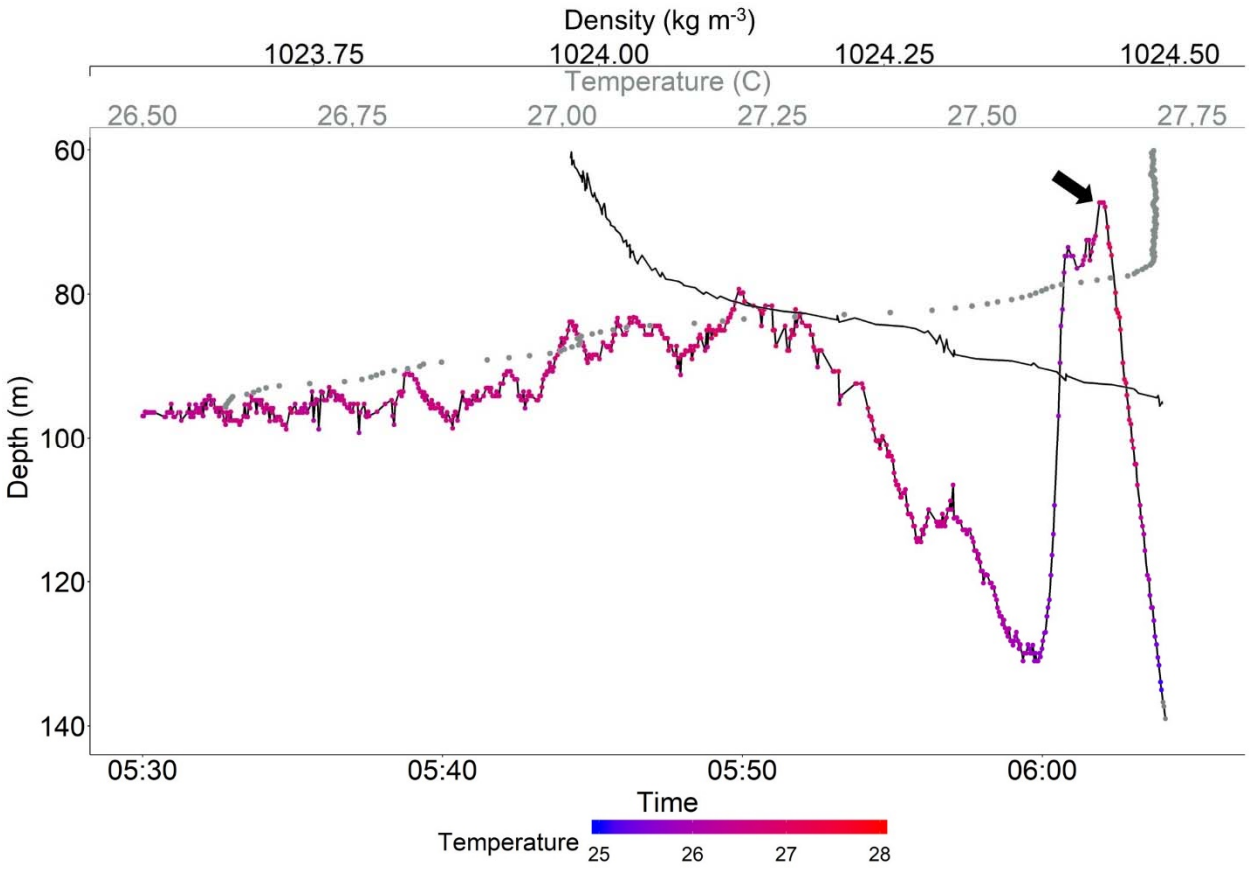


Figure 28. Final two hours of the 2019 dive profile, as portrayed in Figure 1.3. Tag ejection at the pycnocline and thermocline can be seen from 06:02 EST. Colored line depicts ambient temperature of bonefish at depth, black line is CTD density, gray dots are CTD temperature, black arrow indicates tag ejection.

The movement patterns and bonefish physiology provide evidence that the tag expulsion was an observed spawning event. The final descent and ascent happened at a rate seven-times faster than the previous descents and ascents observed during the track, indicative of a spawning rush behavior. The oscillatory movements at 67.3 m lasted for two minutes, and are representative of the "mixing" behavior that is commonly associated with fish spawning in an aggregation (Heyman et al. 2005). The tag expulsion likely coincided with the extrusion of eggs from the cloaca, as bonefish are gymnovarian spawners – their eggs are released from the ovary into the coelomic cavity before being expelled via the oviduct – and thus the eggs and acoustic tag

occupied the same space prior to the spawning event. Furthermore, it has been hypothesized that the porpoising pre-spawn behavior sequesters air into the swim bladder at the surface, and that the diving and spawning rush motion acts as a pneumatic assist to expelling eggs (Danylchuk et al. 2019).

A water column profile was taken to describe the stratification of water masses at the location and time of the spawning event. Upon observing the ejection of the acoustic telemetry tag, a Castaway CTD rated to 100 m was deployed (SonTek YSI, Xylem Inc., New York) (Figure 32). The water column profile revealed a pycnocline and thermocline had established at 75.9 m, which was 7 m below the observed mixing behavior. Spawning at the pycnocline imparts many benefits to larvae, including increased prey availability (Kiørboe et al. 1988; Bjorkstedt et al. 2002; McManus and Woodson 2012) and exploitation of stable conditions within the boundary layer that reduces exposure to turbulent conditions and may also lead to more predictable and stable dispersal mechanisms (Paris and Cowen 2004; Nickols et al. 2012).

These studies in The Bahamas can provide the foundation for a conceptual model in studying bonefish spawning movements, which can then be applied to other populations of *A. vulpes* and potentially other albulid species. Further observations of bonefish diving behavior should be made throughout the Caribbean. Locations with gradually sloping coastal bathymetry, like the Florida Keys, and also nearshore drop-offs like South Abaco and Cape Eleuthera will provide additional perspective on bonefish spawning habitat selection.

Larval dispersal models

Identification and description of the pelagic spawning habitat of bonefish has been an elusive discovery for bonefish researchers despite a decade of effort. Efforts in The Bahamas had resulted in partial descriptions of their offshore movements and diving behavior, while efforts in

the Pacific have been hindered by ocean conditions and remote access to research support. In 2019, our efforts, facilitated by the Fisheries Research Foundation (by providing the M/Y *Albula* research cruise), discovered that bonefish dive to depths reaching 450 feet to spawn. When compared to previous partial tracks of offshore spawning movements in Abaco, The Bahamas, it became apparent that bonefish spawning habitat is variable, and that bonefish move to where they perceive the conditions to be most appropriate for spawning. The fish appear to rely upon identifying the depth of the pycnocline and thermocline, and likely other ocean features such as currents. These discoveries were shared in a presentation at the annual American Fisheries Society meeting by Ph.D. student Steven Lombardo and were published in the journal *Marine Biology* in December of 2020: Novel deep-water spawning patterns of bonefish (*Albula vulpes*), a shallow-water fish (Lombardo et al. 2020).

Larval dispersal simulations of observed spawning events:

This component of the BRRP was created to provide further context and understanding of the new discoveries surrounding Bonefish spawning behavior. For example, spawning depth selection may be explained by density features within the ocean (pycnocline) and thermal features (thermocline) that coincide. Spawning in relation to these features can confer benefits to newly hatched larvae such as food availability, stable environmental conditions, suspension within the water column, and repeatable transport pathways. However, the heterogeneity in offshore spawning migration direction and distance – in the 2013 and 2018 Abaco spawning events, the fish stayed more north, while in 2018 the fish moved south beyond the tip of Abaco – might be explained by sensing acute current patterns. This implies that spawning behavior and habitat selection is influenced by large-scale regularly occurring ephemeral ocean conditions (gyres) and small-scale variation of current conditions within the moment of the spawning event.

Biophysical models – models that combine ocean numerical models of observed conditions and parameterized biological characteristics of organisms of interest – were created to assess larval transport mechanisms and population connectivity. Such models have already been commissioned by BTT (Zeng et al. 2019); however, these models simulated generalized larval transport from known and theorized locations, without biological parameterization of larval development or inclusion of the now likely deep-water origin of bonefish larvae (larvae were simulated with surface transport only). These new biophysical models simulated the fate of bonefish larvae produced from the observed 2019 Abaco spawn, and the partially observed 2013 and 2018 spawns. Results from these models can be applied towards the further assessment of bonefish population connectivity within The Bahamas and may also be used inform future allocation of protective resources. The larval dispersal models have been structured and coded for repeated use as a tool to monitor future bonefish spawns, independent of location with The Bahamas, and can be assessed for the efficacy of use in predicting year class strength.

Ocean observation data sourced from the Navy Coastal Ocean Model American Seas dataset were compiled for 2013, 2018, and 2019. These data were spatially interpolated over a structured grid system that spans the Marquesas, FL, to the West, Mayaguana, The Bahamas, to the East, Cuba to the South, and St. Augustine, FL, to the north, and temporally interpolated to account for gaps in data availability. These interpolated data drive particle dispersal in the biophysical model. Biological parameterization of larval stage bonefish was informed through BRRP discoveries in the bonefish development timeline (Halstead et al. 2020; Mejri et al. 2021) and through descriptions in related taxa – *Anguilla japonica* – by Tuskamoto et al. (2009). These parameters are inclusive of spawning location, depth, and time, buoyancy as a product of pelagic larval duration/development and ocean salinity, diel vertical migration based upon settlement

stage larval behavior (Mojica et al. 1995), and mortality by temperature (Pfeiler 1984; Pfeiler et al. 1988; Sanchez-Velasco et al. 2006; Sachez-Velasco et al. 2013) or pre-settlement stage beaching. Simulations were run 100 times for each spawning event and reflect the fate of 10,000 larvae for each spawning event.

Larval dispersal patterns varied across years; however, 2018 and 2019 shared the most similar dispersal pattern (Figure 29).

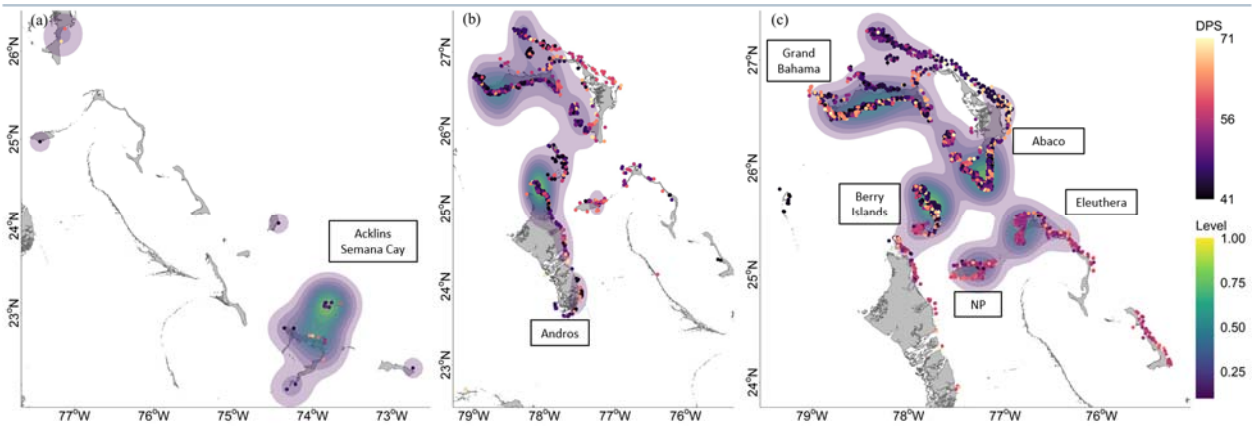


Figure 29. Kernel Density Estimates (KDE) for settled larvae for aggregated LDM iterations. Larvae are colored by settlement days post spawn (DPS), and KDE density estimates (Level) are standardized to a maximum value within a bandwidth of one. Core settlement areas colored yellow. Panels correspond to (a) 2013, (b) 2018, and (c) 2019.

Kernel density estimates for the 2013 spawning event identified that of the 20 larvae that settled out of the total one million, the core settlement area was located southeast from the spawning site, on Semana Cay and Acklins. In 2018, 1190 larvae settled out of the total one million, with core settlement areas to the west and southwest of the spawning site, on western Grand Bahama and north Andros. In 2019, 3790 larvae settled out of the total one million, with core settlement areas in all directions, on south central Grand Bahama, the Berry Islands, New Providence, north Eleuthera, and south Abaco.

Larval connectivity as measured by the percent of simulations that yielded successful settlement and the number of larvae settled indicates that the spawning events in south Abaco most strongly connect to Abaco itself and Grand Bahama (Figure 30).

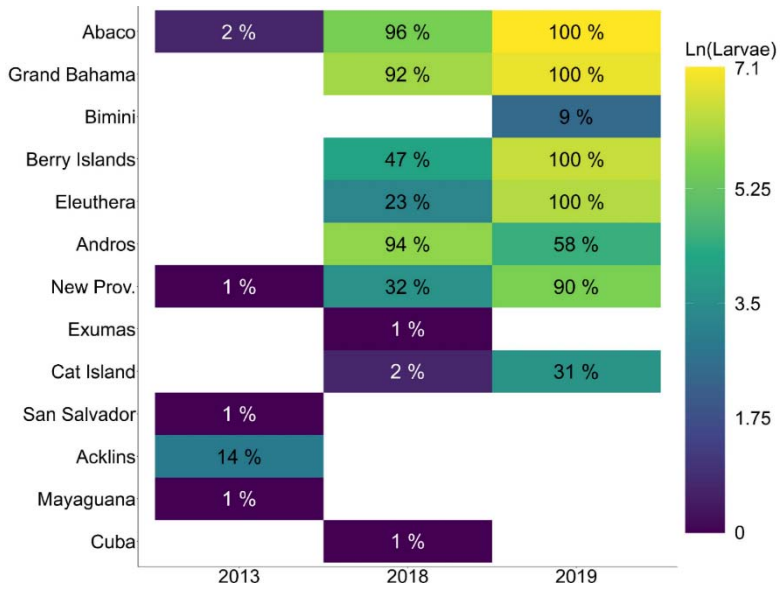


Figure 30. Settled larvae (natural log scaled) per island over the full 71 d larval dispersal period. Count data were aggregated across all LDM iterations. The percent of LDMs that resulted in settled larvae are annotated.

Less consistent, but similarly abundant connections also exist with the Berry Islands, Eleuthera, Andros, and New Providence. The strength of these connections was dictated by the current flow direction at the spawning location, the presence of the winter gyre between Abaco and Grand Bahama, and the presence of smaller eddies that form near the southern tip of Abaco.

The settlement patterns observed through these simulations suggests that national park expansions and additions may be impactful to ensuring the protection of active nursery habitats, though follow up studies to ground-truth the presence of juveniles is necessary. The core settlement areas of the 2018 and 2019 spawning events provide evidence for the following actions: (1) westward expansion of Northshore-The Gap National Park to the western limit of Grand Bahama, (2) merging the Abaco Marls National Reserve and Cross Harbor Protected area to fully encompass the spawning migration route, PSA site, and

nursery habitats, (3) consideration for additional park designations in the north and central region of the Berry Islands and north Andros.

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Intern Projects:

2019. Mason Thurman. Determining Juvenile Bonefish Benthic Habitat Preference using Mesocosms.

2018. Angela Hopkins. Utilization of Lipids During Embryogenesis and Early Life Stage Leptocephalus Larvae in Bonefish

Outreach:

Q & A Session on Instagram Live with All Points Fly Shop & Outfitter • August 2020 • Portland, ME
- Interviewed about current research pertaining to bonefish, tarpon, and permit spawning and movement

Smithsonian Institute World Oceans Day Outreach • June 2019 • Fort Pierce, FL
- Shared PhD research and tools/gear used at the "This is what a scientist looks like" booth

Bonefish & Tarpon Trust Science Blog • April 2019
- Contributed an article detailing the importance of nursery habitat for juvenile bonefish in South Florida

Worldwide Sportsman Bass Pro Shops Seminar Series • April 2019 • Islamorada, FL
- Presented current state of research pertaining to bonefish and habitat use in South Florida and the Caribbean

Press Releases:

- "FAU HARBOR BRANCH PROJECT TO HELP SPORTFISHING INDUSTRY," https://www.fau.edu/newsdesk/articles/BTT_HarborBranch_GrantMar2016.php
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All Points Fly Shop & Outfitter: “Live Q+A with Steven Lombardo – Bonefish spawning, food sources, Biscayne Bay, Bahamas, Tarpon, and more!” (8/2020)

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Appendix

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Bonefish (*Albula vulpes*) oocyte lipid class and fatty acid composition related to their development

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Abstract Bonefish (*Albula vulpes*) are a valuable fishery resource of tropical and subtropical ecosystems worldwide. Despite their importance, there is limited information on bonefish life history and ecology. The present study aims to describe, for the first time, oocytes development and their lipid characteristics in wild bonefish during the reproductive season in different tidal flat locations in Grand Bahama Island, Bahamas. Our results have shown that Bonefish follow group-synchronous ovarian development and produce lipid-rich eggs [total lipid (TL) content was >26% of wet mass (WM)]. The major lipid class was a neutral lipid: the wax esters and steryl esters (WE-SE; >48% of TL), suggesting their use to support buoyancy and/or energy storage. Fatty acid (FA) composition of bonefish oocytes was characterized by high levels of monoenes in the neutral lipid fraction (i.e. 16:1 and 18:1n-9) indicating their probable role as energy fuel. However, the most abundant fatty acids in the polar lipids were docosahexaenoic acid (DHA; 22:6 n-3 > 15% of total polar FA), eicosapentaenoic acid (EPA; 20:5 n-3 > 13% of total polar FA) and arachidonic acid (ARA; 20:4 n-6 > 4% of total polar FA) which were selectively conserved among the tidal flat locations, suggesting their importance as essential constituents of cell membranes during the development of bonefish oocytes. Our results bring useful information concerning the reproductive physiology of bonefish and not only serve as a benchmark for determining the nutrient requirements to produce high quality eggs from bonefish captive broodstock, but also will help establish meaningful management practices for this species.

Keywords Bonefish · Oocytes · Lipid · Fatty acid · Wax esters and steryl esters

Abbreviations

ALC	fatty alcohol
ARA	arachidonic acid
BB	Barbary beach
CA	cortical alveolus
CBE	Crabbing Bay east
DHA	docosahexaenoic acid
EFA	essential fatty acids
EPA	eicosapentaenoic acid
FA	fatty acid
FFA	free fatty acids
HC	hydrocarbon

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KET	ketones
LV	late vitellogenic
MUFA	monunsaturated fatty acids
PL	phospholipids
PG	primary growth
PUFA	polyunsaturated fatty acids
SDWC	South Deep Water Caye
SFA	saturated fatty acids
ST	sterols
TAG	triacylglycerol
TL	total lipids
WE-SE	wax ester - steryl ester
WM	wet mass

Introduction

Bonefishes (*Albula spp*) inhabit shallow tropical and subtropical flats worldwide (Alexander 1961). In the Carribbean, the dominant species on the flats that supports the recreational flats fishery is *Albula vulpes*. Hereafter, Bonefish refers to *A. vulpes*. Bonefish are highly prized sport fish, contributing to a recreational flats fishery with an annual economic impact of $465 million in the Florida Keys (USA) (Fedler 2013), $141 million in the Bahamas (Fedler 2010), and $56 million in Belize (Fedler 2014). Despite their economic value and key ecosystem role, there are considerable gaps in the scientific literature about their reproductive physiology and biology, as well as other features in their life history (Danylchuk et al. 2008).

In the Western Atlantic, it is suggested that bonefish spawn between October and May (Mojica et al. 1995; Murchie 2010; Danylchuk et al. 2011). They form large pre-spawning and spawning aggregations 1–3 days prior to the full moon (Johannes and Yeeting 2000; Danylchuk et al. 2011). Then, the fish migrate to spawn near deep-water drop-offs, off coral reef shelves (Danylchuk et al. 2011).

Characterizing the bioenergetics of egg production of bonefish can yield insights into the role of prey composition and abundance in reproduction of wild populations, and can inform the development of feeding strategies for inducing successful reproduction of captive populations of bonefish. Lipids and fatty acids (FAs) are one of the most important maternal components that affect egg quality in fishes that produce lipid-rich eggs (Sargent et al. 1999a, b, 2002). These nutritional components provide energy reserves and structural components of cellular membranes (Copeman et al. 2002; Tocher 2003). In oviparous species, lipids and FAs are transferred from the female to the oocytes during vitellogenesis (Sargent et al. 1997). It is well documented that lipid and FA profiles of developing eggs (oocytes) can reveal the condition of broodstock and have a critical role in successful early development of marine fish (Harel et al. 1994; Brooks et al. 1997; Rainuzzo et al. 1997; Tocher 2003; Mejri et al. 2017).

Marine pelagic fish eggs contain a wide variety of lipids. Polar lipids, mainly in the form of phospholipids (PLs) are important compounds of membrane lipids that form the double-layered surface of the cells (Wiegand 1996). Neutral lipids include triacylglycerol (TAG) and wax esters-steryl esters (WE-SE), and provide most of the energy consumed by developing embryos (Sargent et al. 1976; Falk-Petersen et al. 1982; Phleger et al. 1997). In addition to being a reserve energy store, WE provide more buoyancy per unit volume because of their lower densities than TAG (Nevenzel 1970). Some studies have also related embryonic development and hatching success to the content of essential polyunsaturated fatty acids (PUFA) namely docosahexaenoic acid (DHA; 22:6 n-3), eicosapentaenoic acid (EPA; 20:5 n-3) and arachidonic acid (ARA; 20:4 n-6), which are essential building blocks in cell membranes and are contained in storage lipids (March 1993; Marteinsdottir and Begg 2002). Furthermore, EPA and ARA are also precursors of eicosanoids, a group of highly biologically active hormones (Howard and Stanley 1999).

Bonefish show high levels of site fidelity during non-spawning season (Boucek et al. this issue and references therein). Therefore, the diet of benthic invertebrates, crustaceans, mollusks, and fishes (Colton and Alevizon 1983; Crabtree et al. 1998) should reflect variable diet quality among bonefish schools that reside on separate flats. These diet differences should be reflected in the concentrations of highly nutritious lipids and fatty acids in bonefish eggs, which influence the fitness of the future leptocephalus larvae (Fuiman et al. 2015). While there is some knowledge of pre-metamorphic lipid dynamics of the leptocephali of bonefish [i.e., bonefish leptocephali lose 50% of their total lipids during metamorphosis, which provides 80% of the energy required (Padrón et al. 1996)], nothing is known about lipid and FA composition of bonefish eggs.

To gain further insight into reproductive physiology of bonefish, the objective of the present study was to investigate oocyte development and the comparative lipid profile and fatty acid composition of oocytes

collected from female bonefish at three tidal flats adjacent to Grand Bahama Island, Bahamas. We investigated the following questions: (1) What is the reproductive state of bonefish when analyzing the developmental stages of the collected oocytes? (2) What is the total lipid, lipid class and fatty acid composition of bonefish oocytes? (3) Is there evidence of lipid retention or conservation, and which lipid classes and fatty acids are of interest? (4) Is there variation in lipid and fatty acid composition across tidal flat locations? We hypothesize that, based on the known high site fidelity of bonefish, there is a spatial variation in food availability and quality in lipid composition of females' oocytes.

Materials and methods

Experimental fish and sampling

Female bonefish were collected from three shallow (< 1 m) flats habitats, along the southern shore of Grand Bahama Isaland, Bahamas; South Deep Water Cay (SDWC, 26°37' N 77°64' W), Barbary Beach (BB, 26°34'N 78°30'W), and Crabbing Bay East (CBE, 26°39' N 77°58' W) during the full moon of February 2017 (spawning season) (Fig. 1). CB and SDWC were separated by 8 km, with BB located 53 and 60 km from CB and SDWC, respectively. Fish were captured on the flats using a 50 m × 1 m beach seine with 2.5 cm mesh. Captured females were first measured for total length, then a soft-tube catheter (Bard 100% latex-free infant feeding tube, 2.27 mm diameter, 26 cm length, attached to a 3 ml syringe barrel) was inserted through the gonopore into the ovary, and 1–2 ml of oocytes were removed. About two thirds of the oocytes were frozen at −80 °C for biochemical analysis and one third were preserved in 10% neutral buffered formalin for histological analysis. All fish were released alive after sampling.

Histological analysis

Oocytes from bonefish females were fixed in Davidson's solution for 48–72 h before being transferred to 70% ethanol for subsequent histological preparation (Barber 1996; Wilson et al. 2005). Then, oocytes were dehydrated in a series of 70–100% ethanol solutions for a minimum of 1 h each. Samples were then clarified in toluene, and embedded in paraffin wax. Multiple 5–8 μm sections were cut from each embedded sample, stained with hematoxylin and eosin, and then mounted on pre-labeled glass slides for examination. Resultant slides were examined at using a compound microscope with a digital image processing system (cellSens, OLYMPUS, Japan).

Biometric analysis

Oocyte diameters were measured with a high-resolution digital microscope (OLYMPUS, SZX7) that uses the Galilean optical system for brilliant, highly resolved images (1x-5.6x zoom range). The developmental stages observed were identified based on the classification of Crabtree et al. (1997). In total 2835 oocytes from 28 fish were measured, staged and randomly counted, with frequencies of each stage expressed as a relative percentage of the total oocytes for each fish.

Lipid and fatty acid analysis

Lipids from a pool of eggs from individual females were extracted according to procedures developed by Folch et al. (1957) and modified by Parrish (1999). The relative proportions of the different lipid classes; ketones (KET), triacylglycerols (TAG), wax esters-steryl esters (WE-SE), free fatty acids (FFA), hydrocarbon (HC), fatty alcohol (ALC), sterols (ST), and phospholipids (PLs) were determined using an Iatroscan Mark-VI analyzer (Iatron Laboratories Inc., Tokyo, Japan) and were developed in a four-solvent system (Parrish 1987, 1999). In addition, 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) and analysed in MSMS scan mode (ionic range: 60–650 m/z) on a Polaris Q ion trap coupled to a Trace GC (Thermo Finnigan, Mississauga, ON, CA) equipped with a Valcobond VB-5 capillary column (VICI Valco Instruments Co. Inc., Broakville, ON, CA). FAME were identified by comparison of retention times with known standards (37 component FAME Mix, PUFA-3, and menhaden oil; Supelco Bellefonte, PA, USA) and quantified with nonadecanoic

Fig. 1 Map showing sampling sites at Grand Bahama Island, Bahamas

acid (19:0) as internal standard. Chromatograms were analyzed using the Xcalibur 1.3 integration software (Thermo Scientific, Mississauga, ON, CA).

Statistical analysis

Variation in oocyte diameter and total lipids by site were tested with one-way analysis of variance (ANOVA) after assumption verification of homoscedasticity and normality with Levene and Shapiro-Wilk tests, respectively. These analyses were performed with the JMP 13 package (SAS Institute Inc., Cary, NC). Permutational analysis of variance (PERMANOVA with 9999 permutations), including a posteriori pair-wise comparisons, was performed on lipid classes and fatty acid profiles and sums of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and PUFA from polar and neutral lipid fractions. Each PERMANOVA was tested with one factor: sites (SDWC, BB, and CBE). Assumptions of multivariate homoscedasticity were verified with a PERMDISP test, and data were transformed (arcsine square root) when necessary. To analyze the similarity in fatty acid profiles among different sites, SIMPER analyses were run using a Bray-Curtis similarity matrix with PRIMER 7 (v. 7.1.12) and PERMANOVA+ (v.1.0.2).

Results

Oocyte stage characteristics

A total of 28 wild female bonefish were sampled; South Deep Water Cay (SDWC, $n = 13$), Barbary Beach (BB, $n = 6$), and Crabbing Bay East (CBE, $n = 9$). Females ranged from 397 to 564 mm total length, without significant differences among sites $t\,(27) = 76.06$, $p = 0.18$. Three reproductive stages were documented in all ovaries (Fig. 2). Primary growth (PG) oocytes and cortical alveolus (CA) stages were present in all ovaries at a percentage of 12 ± 7.5 (mean $\pm$ SD) and $15 \pm 4.4\%$ of total oocytes, respectively. Late vitellogenic oocytes (LV) were present in greatest number in all females ($73 \pm 9.6\%$ of total oocytes) (Fig. 2). The diameter of oocytes at LV stage did not vary between the sites, with an average size of 601 ± 63 μm.

Lipids and lipid classes

Total lipids accounted for 264.78 ± 72.05 mg g^{-1} of the egg wet mass (WM) and did not differ significantly among the sites ($F_{(2,37)} = 2.67$, $p = 0.08$). The major lipid classes in bonefish eggs were wax esters-steryl esters and phospholipids, accounting

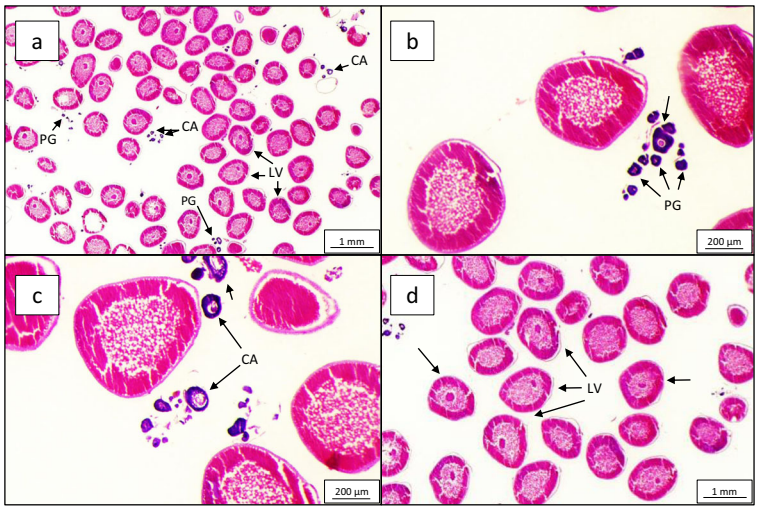

Fig. 2 Micrographs of oocytes from bonefish (*Albula vulpes*) females sampled during the reproductive season (February 2017) in three different tidal flat locations (South Deep Water Cay [SDWC], $n = 13$, Crabbing Bay East [CBE], $n = 9$, and Barbary Beach [BB], $n = 6$) in Grand Bahama Island, Bahamas. **a** wild individual showing oocytes at primary growth (PG), cortical alveolus (CA), and late vitellogenic (LV) stages. **b** primary growth oocytes in the ovary of a wild specimen captured on 13 February 2017. **c** cortical alveolus oocytes in the ovary of a wild specimen captured on 13 February 2017. **d** late vitellogenic oocytes from a wild fish caught on 14 February 2017

for more than 48 and 27% of total lipids, respectively (Table 1). Three of the lipid classes varied significantly among collection sites (*Pseudo-F* $_{(2,\ 37)} = 13.63$, $p = 0.0001$). SIMPER analysis showed that triacylglycerol percentages explained up to 24% of the differences observed between the SDWC, CBE, and BB sites. The percentages of TAG were significantly higher at SDWC (22% of total lipids) compared to BB and CBE, where it was 0.7 and 3.4% of total lipids, respectively.

Fatty acids

Neutral lipid fraction of fatty acids

The fatty acid composition varied according to the flat sites (*Pseudo-F* $_{(2,\ 37)} = 7.63$, $p = 0.0001$) (Table 2). Oocytes sampled from the three flat locations had different fatty acid composition. Overall, in all sites, monounsaturated fatty acids made up the larger fraction (>35% of total neutral fatty acids) compared to

Table 1 Lipid class composition (mean ± S.D., expressed as percentage of the total lipid composition) in bonefish (*Albula vulpes*) oocytes at three tidal flat locations (south Deep Water Cay [SDWC], $n = 13$, Crabbing Bay East [CBE], $n = 9$, and Barbary Beach [BB], $n = 6$) in Grand Bahama Island, Bahamas

Lipid classes	SDWC	CBE	BB
HC	0.50 ± 0.04	0.96 ± 0.47	1.97 ± 1.26
WE-SE	48.27 ± 1.12	52.81 ± 7.22	55.92 ± 1.59
KET	0.21 ± 0.11	0.18 ± 0.12	0.31 ± 0.38
TAG	22.35 ± 0.04^{a}	3.43 ± 0.28^{b}	0.66 ± 0.33^{b}
FFA	0.43 ± 0.00^{b}	2.23 ± 1.40^{a}	0.00 ± 0.05^{b}
ALC	0.00 ± 0.00	0.01 ± 0.06	0.05 ± 0.02
ST	1.27 ± 3.46^{b}	9.39 ± 4.59^{a}	7.95 ± 5.45^{a}
PL	26.96 ± 3.30	31.00 ± 4.43	33.14 ± 2.06
Total lipids ($mg.g^{-1}$)	274.34 ± 74.57	215.84 ± 53.13	295.73 ± 48.28

HC, hydrocarbons; WE-SE, wax esters-steryl esters; KET, ketones; TAG, triacylglycerols; FFA, free fatty acids; ALC, fatty alcohols; ST, sterols; PL, phospholipids. Different letters indicate significant differences among sites

Table 2 Fatty acid composition (mean ± SD, expressed as percentage of total neutral and polar lipids detected) in bonefish (*Albula vulpes*) oocytes sampled at three tidal flat locations (South Deep Water Cay [SDWC], n = 13, Crabbing Bay East [CBE], n = 9, and Barbary Beach [BB], n = 6) in Grand Bahama Island, Bahamas

Fatty acid	Neutral lipid fraction			Polar lipid fraction		
	SDWC	CBE	BB	SDWC	CBE	BB
14:0	3.38 ± 0.95	4.55 ± 0.61	4.96 ± 0.13	1.17 ± 0.25	1.36 ± 0.22	1.10 ± 0.20
15:0	0.80 ± 0.11	1.13 ± 0.10	1.49 ± 0.23	0.74 ± 0.15	0.91 ± 0.16	1.03 ± 0.35
16:0	12.67 ± 2.58	14.41 ± 1.69	14.71 ± 1.03	26.36 ± 2.12	26.07 ± 0.81	23.42 ± 2.00
17:0	1.30 ± 0.26	1.47 ± 0.17	2.47 ± 0.29	1.63 ± 0.34	1.66 ± 0.13	2.26 ± 0.15
18:0	6.09 ± 0.65	6.29 ± 0.91	9.37 ± 2.06	13.48 ± 1.54	14.57 ± 1.37	14.69 ± 0.50
21:0	0.12 ± 0.03	0.13 ± 0.02	0.11 ± 0.00	0.74 ± 0.12	0.68 ± 0.10	0.59 ± 0.06
23:0	0.15 ± 0.05	0.22 ± 0.05	0.09 ± 0.08	0.35 ± 0.12	0.42 ± 0.04	0.19 ± 0.08
24:0	0.18 ± 0.07	0.21 ± 0.05	0.21 ± 0.05	0.64 ± 0.30	0.68 ± 0.16	0.38 ± 0.10
∑SFA$^{\alpha}$	25.53 ± 5.04	29.17 ± 4.24	34.76 ± 4.90	47.53 ± 6.28	50.00 ± 3.77	51.63 ± 12.52
14:1	0.13 ± 0.04	0.19 ± 0.06	0.11 ± 0.02	1.21 ± 0.69	1.51 ± 0.56	0.35 ± 0.05
16:1	16.19 ± 3.85^{a}	17.28 ± 2.37^{a}	7.52 ± 5.51^{b}	3.48 ± 0.81	3.36 ± 0.62	3.57 ± 1.48
17:1	1.59 ± 0.23	1.67 ± 0.33	3.06 ± 0.29	1.24 ± 0.79	1.37 ± 0.13	2.17 ± 1.40
18:1 n-9	23.44 ± 1.71^{a}	25.09 ± 2.52^{a}	20.87 ± 1.14^{b}	3.99 ± 0.80	4.95 ± 0.76	4.52 ± 0.34
20:1 n-9	1.54 ± 0.34	2.08 ± 0.26	3.51 ± 0.00	0.45 ± 0.18	0.48 ± 0.10	0.47 ± 0.17
22:1 n-9	0.89 ± 0.64	0.94 ± 0.54	0.30 ± 0.14	0.79 ± 0.45	0.72 ± 0.28	1.05 ± 0.58
∑MUFA$^{\beta}$	43.89 ± 6.92	47.46 ± 6.26	35.51 ± 18.26	11.91 ± 4.15	13.42 ± 2.62	12.80 ± 4.74
20:2	0.78 ± 0.29	0.58 ± 0.05	0.97 ± 0.71	0.74 ± 0.22	0.70 ± 0.14	0.64 ± 0.15
18:2 n-6	2.41 ± 0.46	2.46 ± 0.44	2.84 ± 0.30	1.56 ± 0.38	1.90 ± 0.12	1.17 ± 0.21
18:3 n-6	0.35 ± 0.14	0.31 ± 0.04	0.10 ± 0.01	0.35 ± 0.15	0.35 ± 0.14	0.36 ± 0.08
18:3 n-3	0.81 ± 0.22	0.69 ± 0.19	1.01 ± 0.11	0.49 ± 0.17	0.49 ± 0.10	0.32 ± 0.16
20:3 n-3	7.22 ± 2.31	4.69 ± 0.63	6.02 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:3 n-6	1.13 ± 0.26	1.15 ± 0.19	1.16 ± 0.13	1.51 ± 0.40	1.96 ± 0.28	1.34 ± 0.10
18:4 n-3	0.54 ± 0.12	0.61 ± 0.11	1.03 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:4 n-6 (ARA)	4.83 ± 2.11	3.65 ± 0.68	4.70 ± 0.68	4.40 ± 1.08	4.10 ± 0.69	3.80 ± 1.44
20:5 n-3 (EPA)	8.11 ± 2.62	5.19 ± 0.58	6.96 ± 0.50	15.21 ± 2.06	13.09 ± 1.62	12.24 ± 3.13
22:6 n-3 (DHA)	4.13 ± 1.23	3.67 ± 0.68	4.31 ± 0.34	15.65 ± 1.82	15.31 ± 1.26	15.06 ± 6.09
∑PUFA$^{\delta}$	30.40 ± 9.93	23.16 ± 3.72	29.52 ± 4.05	39.92 ± 6.29	37.90 ± 4.36	35.19 ± 11.70
n-3	17.52 ± 2.82	13.31 ± 1.88	17.07 ± 2.27	20.54 ± 6.71	19.89 ± 6.56	19.19 ± 6.50
n-6	8.11 ± 3.51	5.19 ± 2.13	6.96 ± 3.01	15.21 ± 7.05	13.09 ± 5.89	12.24 ± 5.66

Different letters indicate significant differences among sites

α: includes 11:0, 12:0, 13:0, 20:0, whose combined percentages are ≤0.2% of total fatty acids

β: includes 15:1, 24:1 n-9, whose combined percentages are ≤0.2% of total fatty acids

δ: includes 22:2, whose combined percentages are ≤0.5% of total fatty acids

saturated fatty acids and polyunsaturated fatty acids (>25 and > 23% of total neutral FAs, respectively). The fatty acid profiles of bonefish oocytes sampled at SDWC and CBE flats were characterized by high levels of oleic FA (18:1 n-9) and FA (16:1) compared to oocytes sampled at BB flat site, as shown by n-MDS analysis (Fig. 3 and Table 2). The two fatty acids explained up to 47% of the differences among sites as determined by SIMPER analysis.

Polar lipid fraction of fatty acids

We observed no effect of sites on fatty acid composition in polar lipids (*Pseudo-F* $_{(2,\ 17)} = 1.35$, $p = 0.2091$).

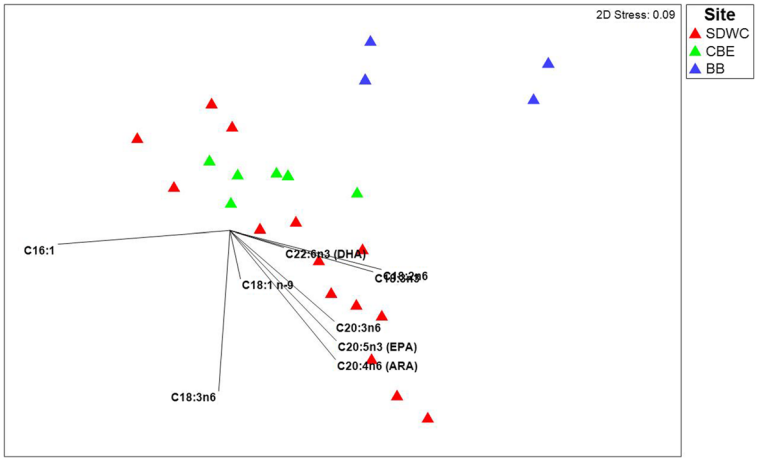

Fig. 3 Non-metric multi-dimensional scaling of the Bray-Curtis similarity matrix based on the relative abundance of neutral fatty acid profiles associated with oocytes sampled from females at three flats sites: South Deep Water Caye (SDWC), Crabbing Bay east (CBE), and Barbary beach (BB). The arrows represent the fatty acid responsible for most of the variation

Percentages of different FAs at the three different sites examined are presented in Table 2. Here, saturated fatty acid and polyunsaturated fatty acid percentages made up the larger fraction (>47 and > 35% of total polar FAs, respectively; Fig. 4 and Table 2) compared to monounsaturated fatty acids (>12% of total polar FAs). The highest essential FA concentrations were DHA (22:6 n-3) followed by EPA (20:5 n-3) and ARA (20:4 n-6; Table 2).

Discussion

Reproductive behavior

Female bonefish exhibited three stages (clutches) of oocytes: the dominant clutch is late vitellogenic stage, a second clutch is likely 'arrested' in the cortical alveoli (yolk vesicle) stage, and a third clutch of non-yolky oocytes in the primary growth phase. This pattern allows for multiple, distinct ovulatory events that typically follow seasonal, lunar, or diurnal cycles (Parenti and Grier 2004), which suggests that bonefish follow a group-synchronous ovarian development. Group synchronous fish have two or more distinct populations of oocytes present at the same time and ovulate once in a season, or undergo multiple ovulations over a few days or weeks within the spawning season (Parenti and Grier 2004). This is the most common ovarian type among teleost fishes (Asturiano et al. 2002; Murua and Saborido-Rey 2003) and has been documented in white mullet (*Mugil curema*) (Solomon and Ramnarine 2007), European sea bass (*Dicentrarchus labrax* L.) (Carrillo et al. 1989; Mayer et al. 1990) and tucunare (*Cichla kelberi*) (Normando et al. 2009). Recent studies have shown that females of these species can have up to four ovulations during the natural reproductive period (Asturiano et al. 2000). Thus, one bonefish female

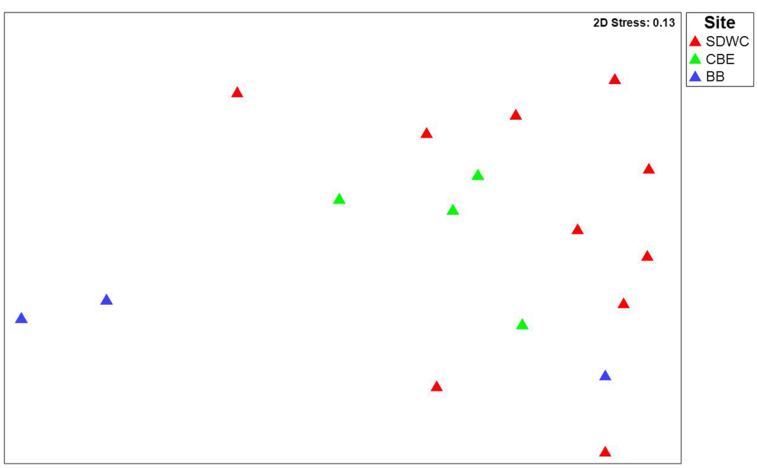

Fig. 4 Non-metric multi-dimensional scaling of the Bray-Curtis similarity matrix based on the relative abundance of polar fatty acid profiles associated with oocytes sampled from females at three flats sites: South Deep Water Caye (SDWC), Crabbing Bay east (CBE), and Barbary beach (BB)

may ovulate more than once during the extended (November through April) reproductive season.

Lipids and lipid classes

Bonefish have lipid-rich eggs containing more than 26% of their wet mass in lipids. Fish eggs can be classified into two energetic categories according to their lipid characteristics (Mourente and Vázquez 1996); eggs with high (> 15% of egg WM) or low (<15% of egg WM) lipid content. The high lipid content in eggs of some fish species as compared to others may arise from different energy needs of the fertilized eggs (i.e., duration of embryogenesis and length of endogenous feeding) (Mourente and Vázquez 1996). The relatively high lipid concentration in bonefish eggs thus suggests high energetic requirements for embryonic and pre-feeding larval development.

Lipids within bonefish eggs from all sites were mainly composed of WE-SE (> 48% of total lipids) and PLs. The WE is the dominant lipid class of eggs and gonads from many marine fish species, including European sea bass (Navas et al. 1997), mullet (Spener and Sand 1970), striped bass (*Morone saxatilis*) (Eldridge et al. 1983), and golden perch (Anderson et al. 1990). Wax esters have three possible functions when present in marine organisms; as a reserve energy store, as a buoyancy agent, and as a structural element (Nevenzel 1970). There are indications that WE have slightly lower densities than TAG, and thus provide more buoyancy per unit volume (Nevenzel 1970). Possibly, the WE-SE, in contrast to TAG, are not subject to hormone-controlled mobilization during periods of energy demand, thus stabilizing the buoyancy of the organism against short term fluctuations (i.e., buoyancy control is divorced from energy demand) (Bogevik 2011). The high concentration of WE-SE in bonefish oocytes suggests that the eggs of this species could be positively buoyant, which would assist eggs spawned at depth to rise to the surface. During the reproductive season, bonefish from Western Atlantic move from their typical shallow water flats to deep waters overlying coral reef edges to spawn (Danylchuk et al. 2008; Danylchuk et al. 2011). It has been shown some fish species rich in WE-SE (eggs and muscles) undertake daily vertical migrations of 300 m or more; during such movements pressure changes of 30 atm are experienced, with WE-SE lipids thought to play an important role as constituents in osmotic regulation and buoyancy control (Bogevik 2011). For instance, eggs of *Merluccius paradoxus*, *M. hubbsi*, and *M. capensis* are positively buoyant because of their high amount of WE-SE (>17% of total lipids) (Kayama and Hirata 1986; Olivar and Fortuno 1991; Sundby et al. 2001).

Bonefish mark-recapture data (Boucek et al., this issue and references therein) reveal high site fidelity of this species. Our results showed significant differences in lipid classes (TAG, FFA, and ST) among oocytes from three different shallow water sites within 8 to 66 km from one another. TAG was higher in oocytes from SDWC site (22% of total lipids) than BB and CBE sites (< 4% of total lipids). The benefits of greater TAG content in bonefish oocytes from SDWC site are likely to be advantageous for the future larvae due to the potential role of TAG as energy reserves. Hydrolysis of TAG is significantly faster than WE-SE, from 1 to 2 orders of magnitude to four- fivefold dependent on species (Bogevik 2011). Indeed, Padrón et al. (1996) have shown that TAG was the principal lipid class broken down during metamorphosis in bonefish leptocephali. It has been observed that decreases in TAG over the spawning season in oocytes of the striped trumpeter (*Latris lineata*) was indicative of decrease in egg quality as maternal resources diminish over time (Bransden et al. 2007; Bachan et al. 2012). Thus, females from SDWC site may have access to better feeding resources comparing to the two other sites (BB and CBE), and increased portion of TAG in eggs may, therefore, provide larvae from SDWC with a potential survival advantage during the transition to exogenous feeding.

The results on the other lipids are less conclusive. The variability of FFA is probably too low with levels of 0% in BB to 2.2% in CBE to have a biological effect. High FFA is an indicator of lipid degradation, otherwise it is undoubtedly a metabolic intermediate (Parrish 1999), but as no difference in oocyte development was observed between sites, there are probably not related to biological activity. Sterol is an essential component in animal membranes, with multiple effects on their physical properties including membrane fluidity, phase behavior, thickness, and permeability (Crockett 1998). Levels in ST were the lowest in SDWC site (1.2% comparaed to over 8% in other sites). Clearly, the oocytes from SDWC seem less thick or less fluid. However, this needs to be further investigated to see if there are differences in water salinity and/or temperature between sites that contribute to these differences. The absence of site effect on WE-SE composition in

bonefish oocytes indicates that the provisioning of this lipid may be tightly regulated in bonefish, however this remains unknown and warrants further investigation.

Fatty acids

Fatty acids mobilized by female fish during gonadogenesis are transferred via serum vitellogenin to developing eggs in the ovary. Thus, the essential fatty acids, vital for early survival and development of newly hatched larvae, are determined by the lipids derived directly from the dietary input of the female during the period preceding gonadogenesis (Kjørsvik et al. 1990; Sargent et al. 1995). In our study, neutral FAs varied among the three sites, where we observed higher percentages of MUFA (i.e, 18:1 n-9 and 16:1) at SDWC site. Given the high site fidelity of bonefish, this suggests that a possible spatial variability in food availability and/or quality that could affect the egg quality and the future larval survival. For instance, levels of essential fatty acids in gilthead sea bream and red drum eggs were found to be closely tied to recent diet (Harel et al. 1994; Fuiman and Faulk 2013), suggesting that some fishes may undergo spawning migrations to incorporate nutrients into their eggs that are specifically available at the spawning site (Fuiman and Faulk 2013). Bonefish may fall into this category where they allocate nutrients available in their tidal flat locations to the eggs a few hours or days before spawning.

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). Our results showed that both the sums of SFA and PUFA were particularly high in the polar lipid fraction of bonefish oocytes accounting for more than 47 and 35% of total fatty acids, respectively, which fall within the published range for early-metamorphosing bonefish leptocephali (Padrón et al. 1996). Indeed, all classes of fatty acids (SFA, MUFA, and PUFA) were utilized and contributed to energy production during metamorphosis with a selective conservation of DHA (Padrón et al. 1996). Clearly, the higher levels of DHA present in bonefish oocytes in our study would allow more DHA to be incorporated into vitally important neural membranes, thereby enhancing physiological function and survivability.

We found that two other essential fatty acids dominate bonefish eggs: EPA and ARA. These EFAs have been identified as being important to a variety of functions in various marine fish species, including survival (Bessonart et al. 1999; Arendt et al. 2005), growth (Shields et al. 1999; Wacker and Von Elert 2001; Bell and Sargent 2003; Copeman and Laurel 2010), sensory and nervous system function (Shields et al. 1999), stress tolerance (Koven et al. 2001; Montero et al. 2003) and ecological performance (Ishizaki et al. 2001; Fuiman and Ojanguren 2011). Arachidonic acid, which accounted for >4% of total FAs in both lipid fractions, is known as the major eicosanoid precursor in fish cells, including prostaglandins, thromboxanes and leukotrienes, among others (Bell et al. 1994; Van Der Kraak and Biddiscombe 1999). These metabolites are important in the control of ovulation and are probably involved in embryonic development of the immune system, hatching and early larval performance (Mustafa and Srivastava 1989; Wade and Van Der Kraak 1993).

Conclusions

Although many studies have examined with changes in lipid class and fatty acid composition during early development in teleost fishes, no previous data are available for developing eggs from fishes in the subdivision Elopomorpha, which includes bonefishes, tarpons, and true eels, a group that has have unique larvae called leptocephali. This study indicates that bonefish likely follow a group-synchronous ovarian development and produce lipid-rich eggs. The eggs are particularly rich in wax esters-steryl esters, which suggest that this lipid class is used for buoyancy and/or energy reserves. Levels of neutral lipids (TAG and MUFA) varied based on the tidal flat locations. Thus, the lower TAG percentages in bonefish oocytes from BB and CBE sites may be indicative of either poorer feeding conditions experienced by bonefish females on the individual flats, or the fish were at a different developmental stage relative to spawning (i.e., the SDWC fish may have been getting ready to spawn in the next lunar phase, whereas the BB and CBE fish would spawn in a future lunar phase). More comprehensive sampling over large spatial and temporal scales is needed to better address these differences. Moreover, the results demonstrate the importance of EFAs (DHA, EPA, and ARA) that were selectively conserved among the sites, suggesting their importance during the ontogeny of bonefish. Finally, analyses of lipids and fatty acids in wild-caught oocytes are a useful tool for the assessment of egg quality and larval viability

of bonefish. These findings highlight that tidal flat location, habitat quality, prey availability, and similar factors could be important determinants for nutrients incorporated into the eggs, which in turn is crucial for egg and larval development and survival. Thus, this should be further investigated and must be considered in the management of this important fisheries resource.

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Compliance with ethical standards

Ethical approval The experimental protocol received approval from the Florida Atlantic University's Institutional Animal Care and use Committee (IACUC, protocol A16–34).

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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

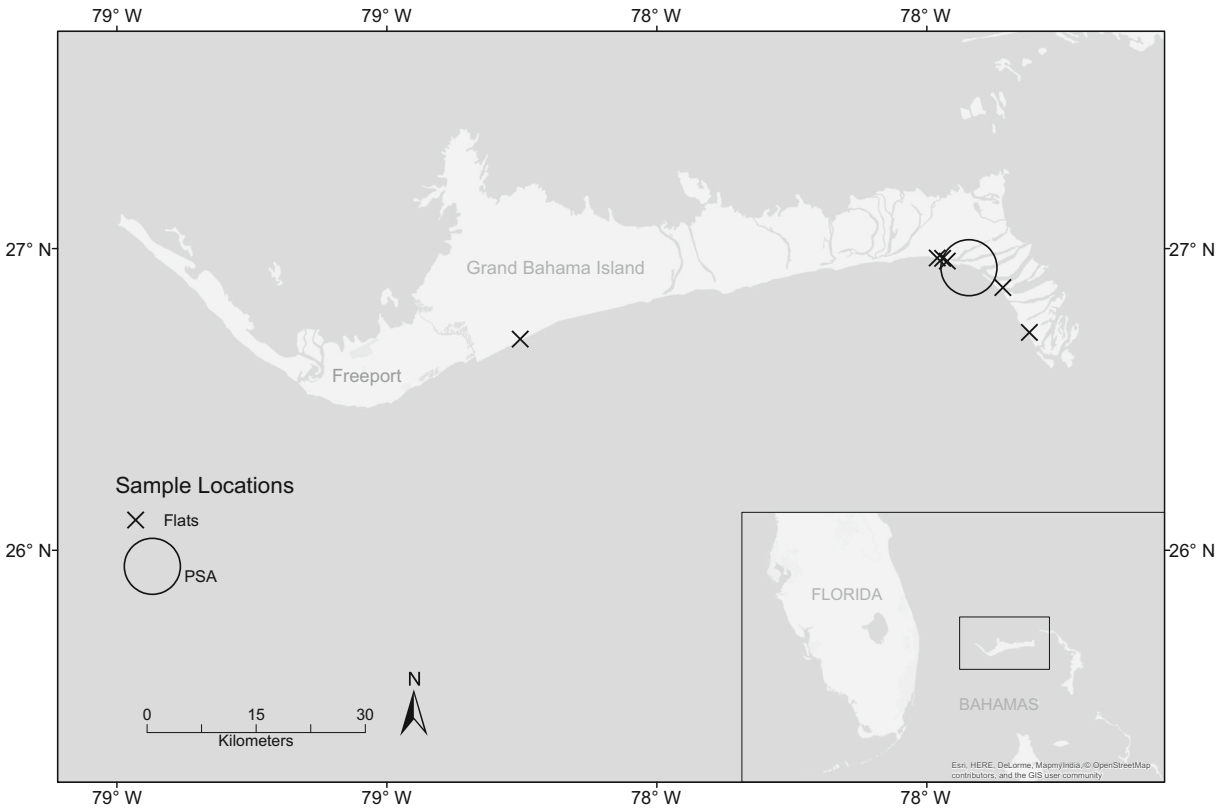

Fig. 1 Map showing sampling locations for both flats and pre-spawn aggregation (PSA) sites during spawning (February, March, and April 2017) and non-spawning (September 2017) months of bonefish (*Albula vulpes*) in Grand Bahama Island, Bahamas. Exact PSA location has been generalized for conservation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

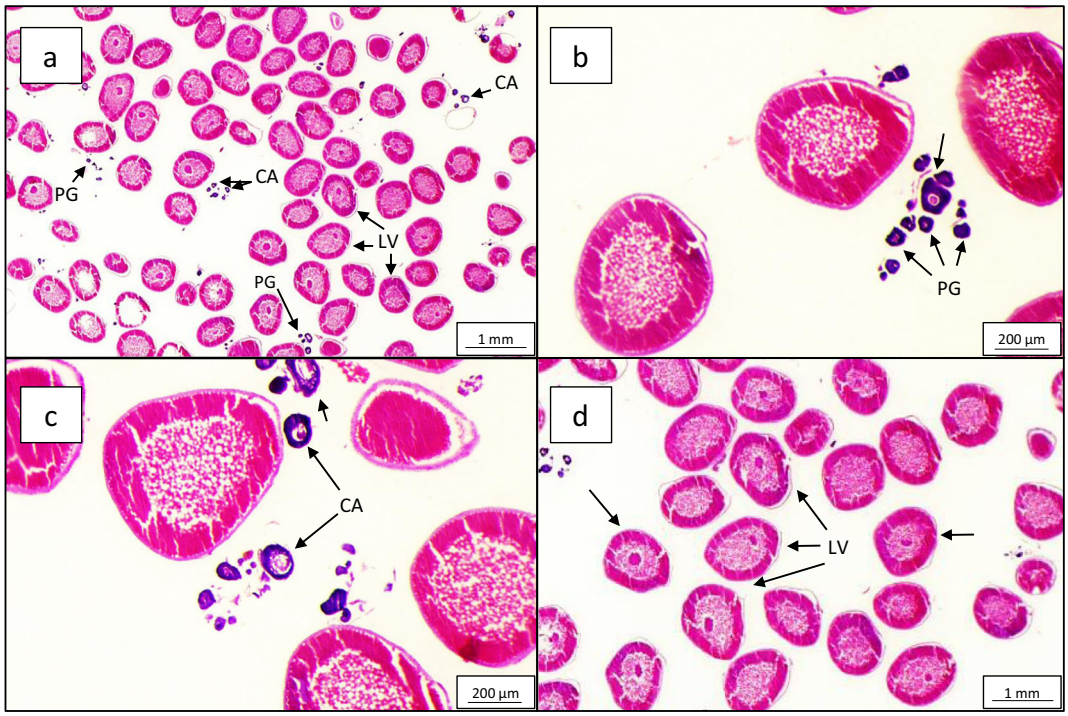

Fig. 2 Micrographs of histologically sectioned oocytes sample collected from a wild female bonefish (*Albula vulpes*) on a shallow water flat during full moon (April 2017). **a** three different developmental oocyte stages are present: **b** primary growth (PG), **c** cortical alveolar (CA), and **d** 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 ($q = 4.247$, $df = 1$, $p = 0.825$).

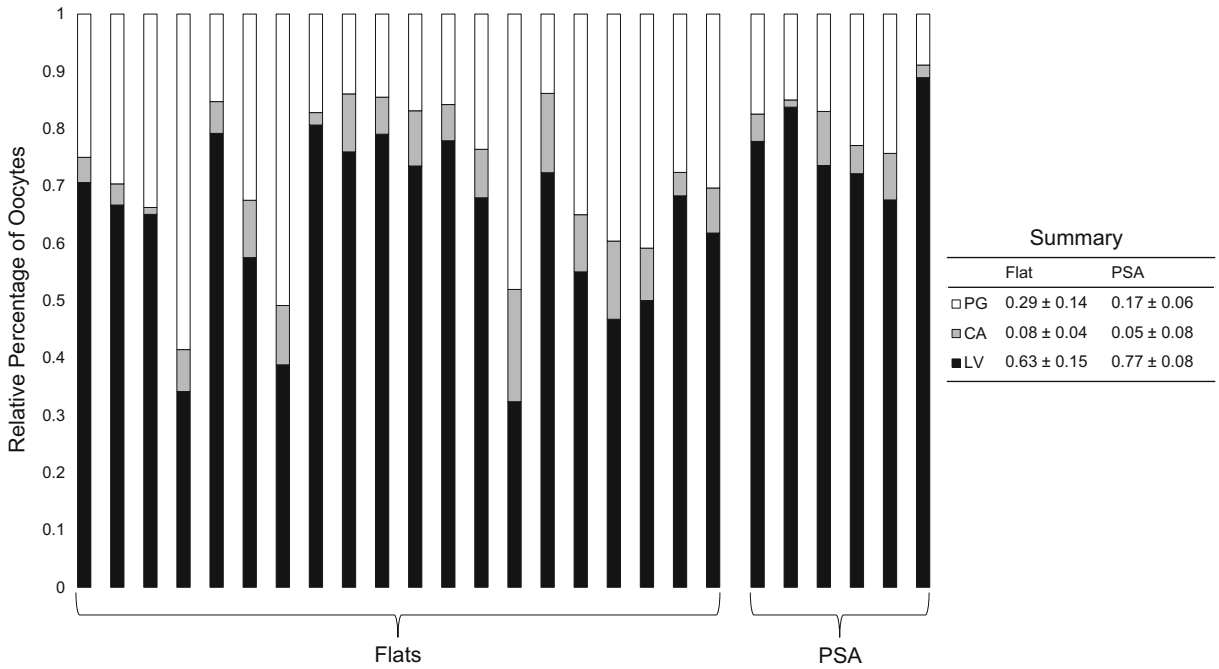

Fig. 3 Proportional occurrence for each of the three oocyte stages: late vitellogenic (LV), cortical alveolar (CA), primary growth (PG) at the two sites (flats and pre-spawn aggregation [PSA]) sampled during the spawning month of April 2017. Oocytes were collected from sexually mature bonefish (*Albula 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

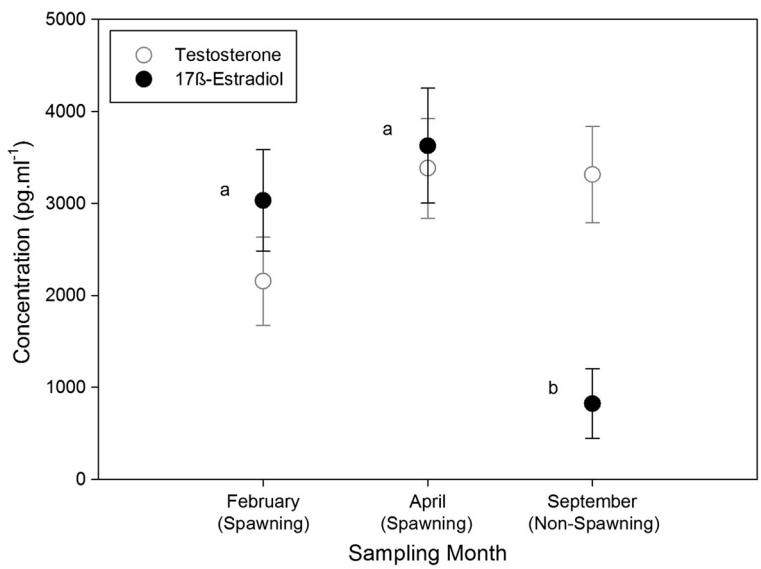

Fig. 4 Monthly plasma concentrations of 17β–estradiol and Testosterone (mean ± SEM) from bonefish (*Albula vulpes*) females sampled in the flats during spawning (February and April 2017) and non-spawning (September 2017) months (mean ± SEM). Letters indicate significant differences in levels of 17β –estradiol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

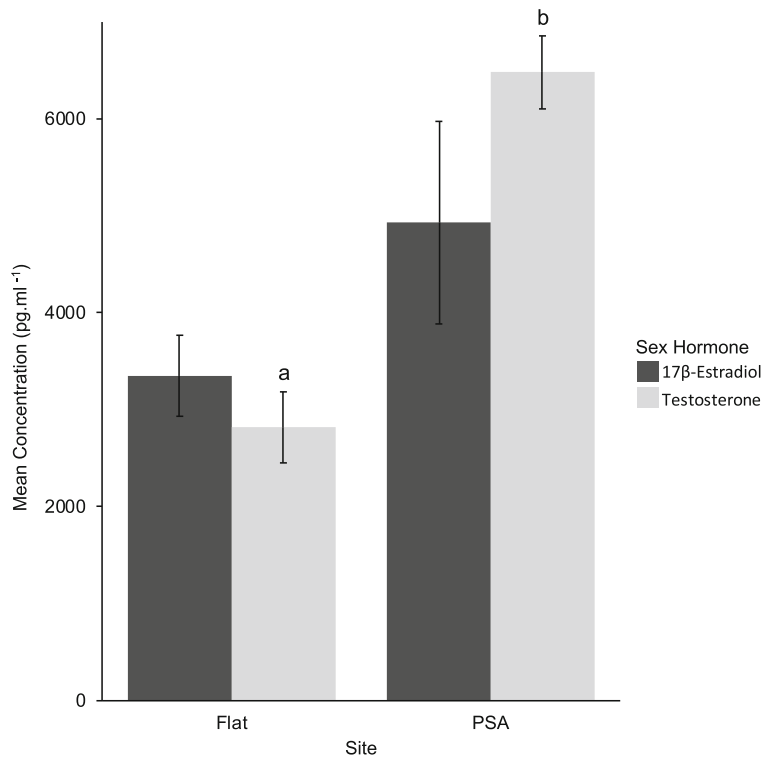

Fig. 5 Plasma concentrations of 17β –estradiol and testosterone (mean ± SEM) at flats and pre-spawn aggregation (PSA) sites in female bonefish (*Albula vulpes*) sampled during three spawning months (February, March, and April 2017). Letters indicate significant differences in Testosterone levels between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

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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

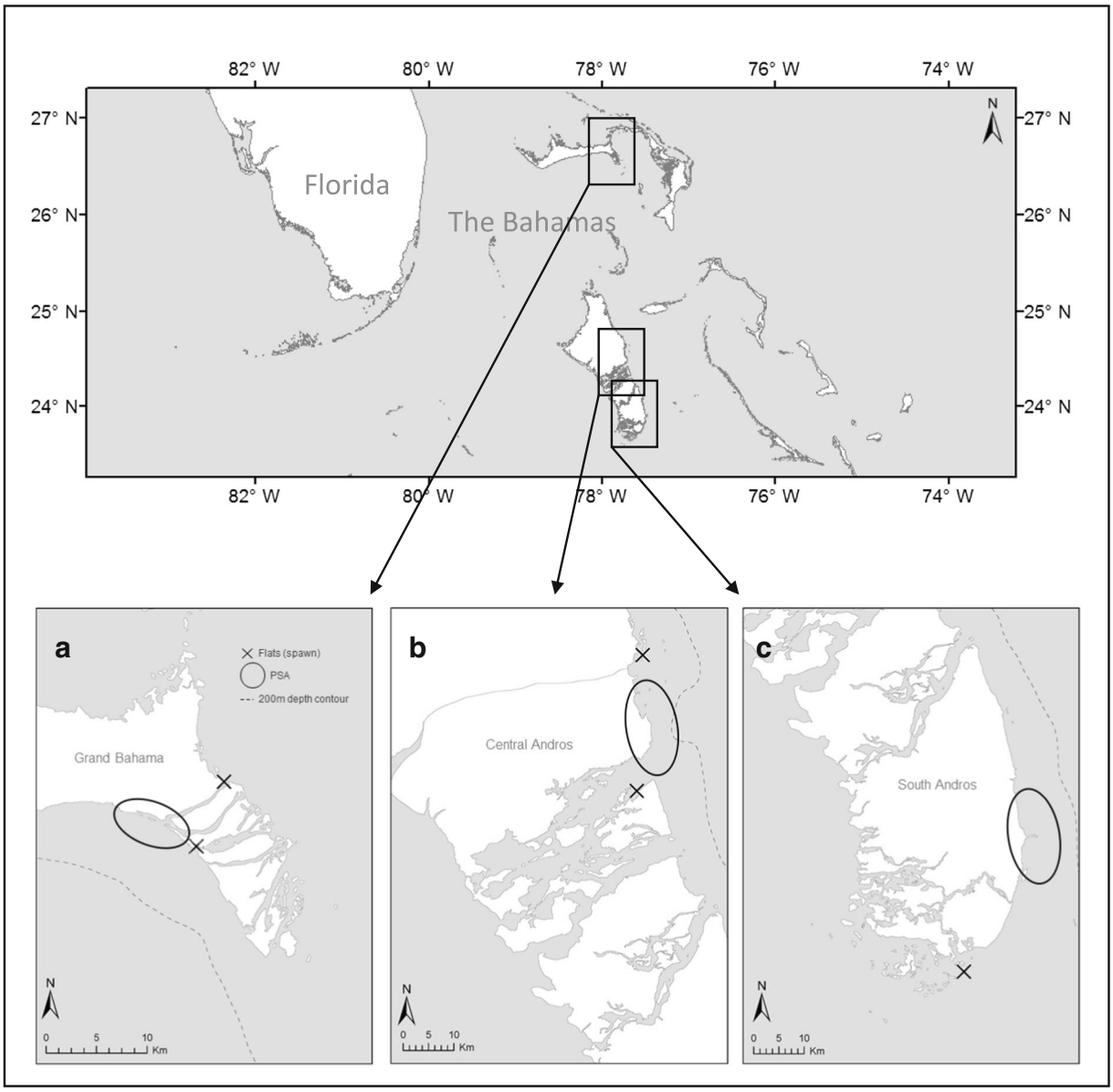

Fig. 1 Map showing sampling locations across three islands in The Bahamas, and the respective flats and pre-spawn aggregation (PSA) habitat sampled within each. Flats habitat was sampled during both spawning and non-spawning months and has been labeled as flats (spawn) and flats (non-spawn) respectively. 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

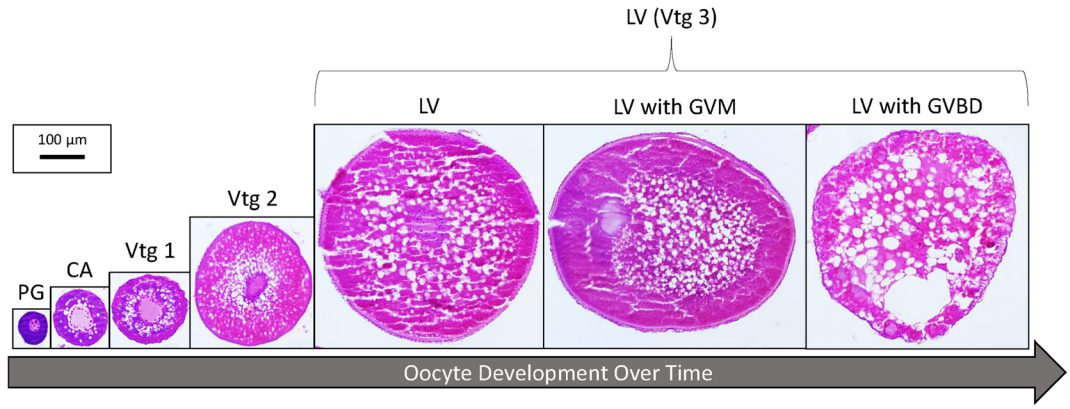

Fig. 2 Chronological schematic of oocyte stages present during the reproductive development of bonefish (*Albula* spp.) including primary growth (PG), cortical alveolar (CA), primary vitellogenic (Vtg 1), secondary vitellogenic (Vtg 2), tertiary vitellogenic (LV), LV with germinal vesical migration (GVM), 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

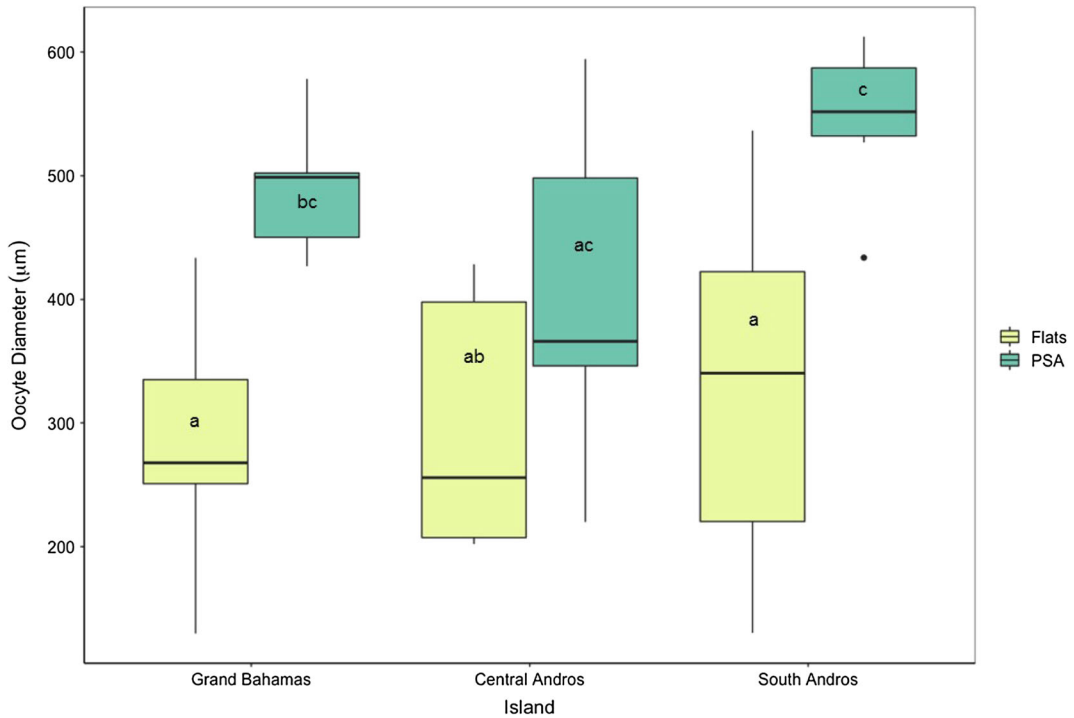

Fig. 3 Diameters of vitellogenic oocytes collected from female bonefish (Albula spp.) sampled at two different habitats (flats and pre-spawning aggregation [PSA]) across three different islands (Grand Bahama, Central Andros, and South Andros, The Bahamas). The top and bottom borders of boxes indicate 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

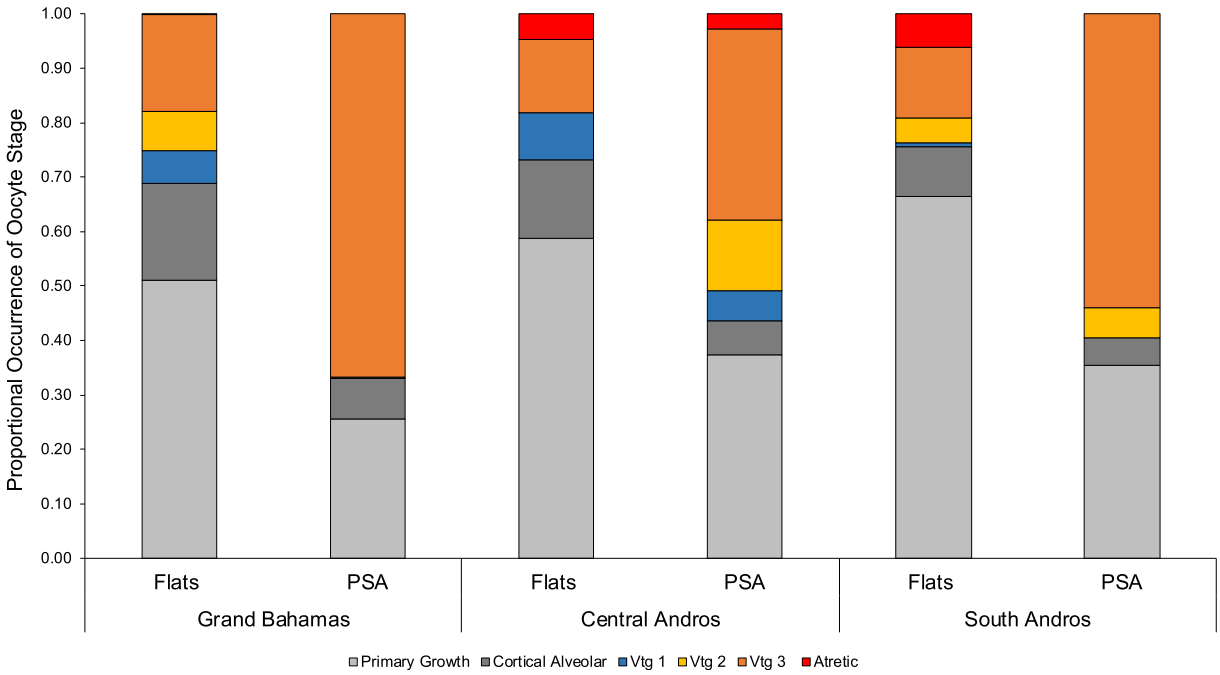

Fig. 4 Proportional distribution of primary growth (PG), cortical alveolar (CA), primary vitellogenic (Vtg 1), secondary vitellogenic (Vtg 2), tertiary vitellogenic (Vtg 3), and atretic oocytes found in female bonefish (*Albula* spp.) sampled from both the flats and PSAs of Grand Bahama, Central Andros,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

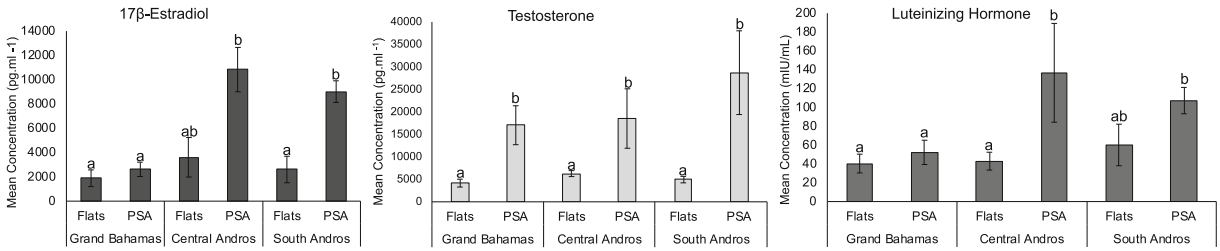

Fig. 5 Spatial variation in 17β-estradiol, testosterone, and luteinizing hormone concentrations (mean ± SEM) for bonefish (*Albula* spp.) sampled across three islands (Grand Bahama, Central Andros, and South Andros, The Bahamas) and two habitat types (flats and PSA) within each island. Black letters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

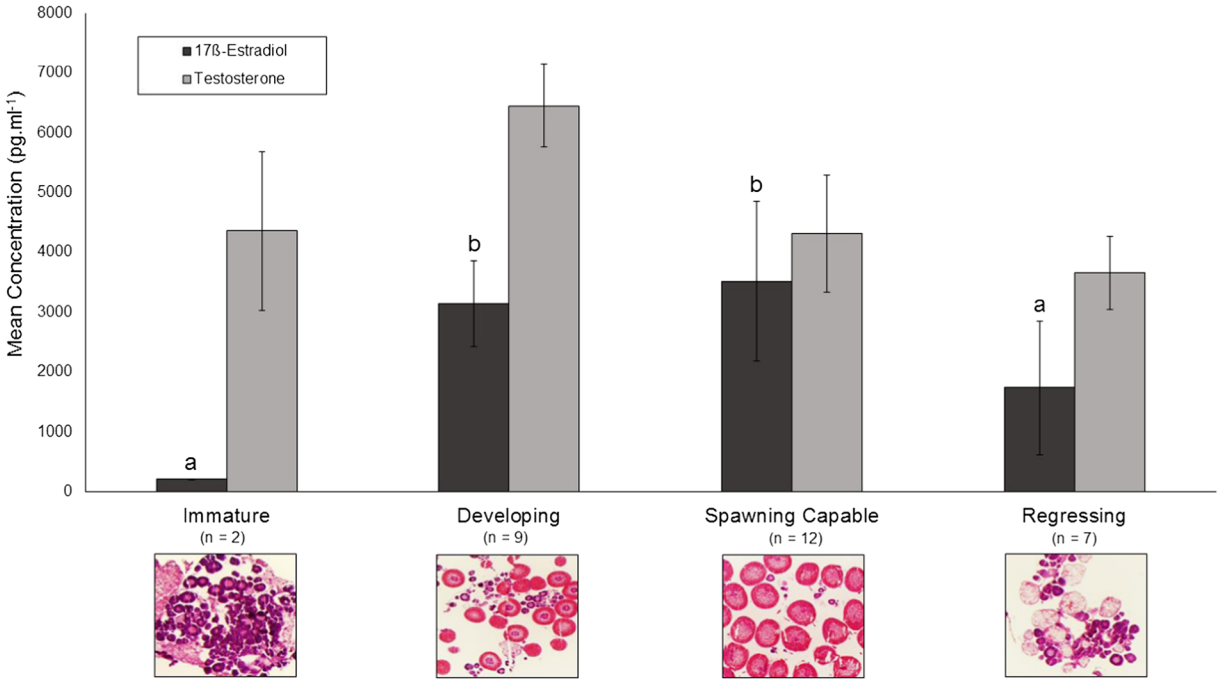

Fig. 6 Concentrations of 17β-estradiol and testosterone (mean ± SEM) for each reproductive phase observed in female bonefish (*Albula* spp.) sampled on flats across all islands (Grand Bahama, South Andros, and Central Andros, The Bahamas). Letters indicate significant differences in mean concentration 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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Place, and for the extensive knowledge provided by Justin Lewis. The experimental protocol for this study received approval from Florida Atlantic University's Institutional Animal Care and Use Committee (Animal Use Protocol #A16-34). HBOI-FAU's IACUC committee follows the animal welfare guidelines in the National Research Councils "Guide for the Care and Use of Laboratory Animals, 8th Edition" National Academy Press Washington D.C. 2011. In addition, we follow the American Fisheries Society's "Guidelines for the Use of Fishes in Research" American Fisheries Society, Bethesda, M.D. 2014. HBOI-FAU's animal care facilities are accredited by American Association for the Accreditation of Laboratory Animal Care (AAALAC).

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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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SHORT COMMUNICATION

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A novel attempt at artificial spawning of captive bonefish (*Albula* spp.)

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Bonefish (*Albula* spp.) are tropical and sub-tropical marine fish that inhabit shallow habitats in the Western Atlantic and Caribbean Sea and typically spawn around full or new moons from October to April (Danylchuk et al., 2011). They are a highly prized sportfish contributing to a recreational flats fishery with an annual economic impact of $465 million in the Florida Keys, USA, (Fedler, 2013) and $141 million in the Bahamas (Fedler, 2010). Bonefish populations in the Florida Keys and in numerous locations throughout the Caribbean are in decline (Brownscombe et al., 2019; Santos et al., 2017), which has led to a 'Near Threatened' listing by the IUCN (Adams et al., 2012) and a need for more research.

Breeding bonefish in captivity provide a novel complement to existing scientific knowledge, allowing for research insights into poorly understood early life stages as well as an important first step for establishing the feasibility of a potential restoration program through stocking. Anguilliformes share similarities in spawning and reproductive biology with bonefish, such as the unique leptocephalus larval stage. The successful spawning of captive eels through environmental and hormone manipulation in both Japan and Europe (Ohta et al., 1997; Sørensen et al., 2016), provides evidence that similar achievements may be possible with bonefish.

This study focuses on the use of captive breeding techniques to spawn bonefish (*Albula* spp.) in captivity. We aimed to (a) induce gonad maturation and spawning in captive bonefish, (b) monitor hormone levels related to maturation (testosterone and 17β-Estradiol) in captive bonefish and (c) assess the development and nutrient composition (fatty acids [FAs] and free amino acids [FAAs]) of oocytes collected from captive bonefish.

In our Florida laboratory, a combination of environmental manipulation (i.e. photoperiod and temperature) and hormone injection was used to simulate ambient conditions for captive bonefish. In The Bahamas laboratory, fish were reared under ambient photoperiod and abiotic conditions. Wild adult bonefish (*Albula vulpes*) were collected in 2016 from Biscayne Bay (BB), FL, (n = 23; Fork Length (FL); 474 ± 54 mm, Weight; 1,247 ± 482 g) and the Middle Keys (MK), FL, (n = 21; FL; 408 ± 50 mm, weight; 1,828 ± 724 g). Fish, collected from BB and MK, were held in captivity for 1 year at the Harbor Branch Oceanographic Institute (HBOI) aquaculture park, Florida, USA. Fish were held in a 12,000 L tank at the HBOI aquaculture park, Fort Pierce, Florida, USA and were fed White shrimp (*Litopenaeus setiferus*), Pacific krill (*Euphausia pacifica*) and Blue crab (*Callinectes sapidus*) daily. In an attempt to induce gonad maturation in captivity, the rearing environment was manipulated to mimic natural seasonal changes in water temperature in the Florida Keys, by placing the fish on a 4- and 6-month abbreviated photo-thermal cycle in March 2018 (Table 1).

William R. Halstead and Cameron Luck contributed equally to the manuscript.

Hormonal stimulation with carp pituitary extract (CPE, Stoller Fisheries, Table 1) started in December 2017 and February 2018 for MK and BB fish, respectively. Because bonefish are a sexually monomorphic species, gender and reproductive status were only possible to assess using palpation, cannulation and ultrasound at sexual maturation and only data from identified females (five females from each location, a total of 10) are presented here (Table 1). Doses of CPE were characterized as very low dose (VLD, 5 mg/kg), low dose (LD, 10 mg/kg) and high dose (HD, >20 mg/kg). Females 1, 2 and 3 received no CPE injections in 12/2017, then received VLD in 01/2018 followed by LD in 02/2018 (n = 3). After short cycling, the same females received either no injections (n = 1)

TABLE 1 Captive bonefish (*Albula vulpes*) female hormonal treatment schedule. Fish were kept in captivity for over 1 year with no hormonal injections (NHI) at Harbor Branch Oceanographic Institute (HBOI) aquaculture park, Florida, USA

Origin	Female ID	Hormonal Manipulations									
		Date (Month/Year)									
		01-17 until 11-17	12-17	01-18	02-18	03-18	04- 18	05- 18	06-18	07-18	08-18
Middle Keys	1	NHI		5	10	4-month abbreviated photo-thermal conditioning cycle schedule				20	20
	2	NHI		5	10					20	20
	3	NHI		5	10					NHI	NHI
	4	NHI	10	10	10					30	30
	5	NHI	10 o	10 o	10 o					20 o	20
Biscayne Bay	6	NHI			5 o	6-month abbreviated photo-thermal conditioning cycle schedule					
	7	NHI			5 o						
	8	NHI			5						
	9	NHI			30 x 2 o						
	10	NHI			30 x 2 o						

Week	Light/Dark	Temperature (!C)
1	10.5:13.5	26
2	11:13	27
3	12:12	28
4	13:11	29
5 to 10	14:10	30
11	13:11	29
12	12:12	28
13	11.5:12.5	27
14	11:13	26
15	10:14	25
16	9:15	23-24

Week	Light/Dark	Temperature (!C)
1 to 2	10.5:13.5	26
3	11:13	27
4	11.5:12.5	27
5	12:12	28
6	12.5:11.5	28
7	13:11	29
8	13.5:10.5	29
9 to 15	14:10	30
16	13.5:10.5	29
17	13:11	28
18	12.5:11.5	28
19	12:12	27
20 to 21	11:13	26
22	10.5:13.5	25
23	10:14	25
24	9:15	23-24

Note: Hormonal stimulation started in December 2017 for Middle Keys fish and in February 2018 for Biscayne Bay fish, respectively. Females received an intraperitoneal injection with Carp Pituitary Extract at different doses (CPE, Stoller Fisheries). CPE injections were characterized as very low dose (5 mg/Kg), low dose (10 mg/Kg), and high dose (>20 mg/Kg). Nested tables represent short and long photo-thermal conditioning cycles schedule for female bonefish. o: Indicates presence of oocytes during cannulation. 5, 10, 20 and 30: refer to CPE doses per kg fish. x 2: Indicates fish were injected twice. Second dose injected 24 to 48 h following the first dose.

TABLE 2 Captive bonefish (*Albula* spp.) female blood and oocyte collection schedule at Cape Eleuthera Institute (CEI), Eleuthera, The Bahamas

		Blood and oocyte collection									
		Date (Month/Year)									
Origin	Female ID	06–17	07–17	08–17	09–17	10–17	11–17	12–17	01–18	02–18	03–18
CEI	1	+						+ o	+ o	+ o	+ o
	2	+						+	+	+ o	+
	3	+						+	+ o	+ o	+ o
	4	+						+	+ o	+	+ o

Note: Tanks (n = 4) were exposed to ambient photoperiod and temperature for 11 months (April 2017–March 2018). Fish were squeezed, cannulated and blood samples were collected monthly, starting in June 2017, to determine gender and evaluate gonad development. No hormone injection trials were conducted on these fish. o: Indicates presence of oocytes during cannulation. +: Indicates blood sample was collected from the female.

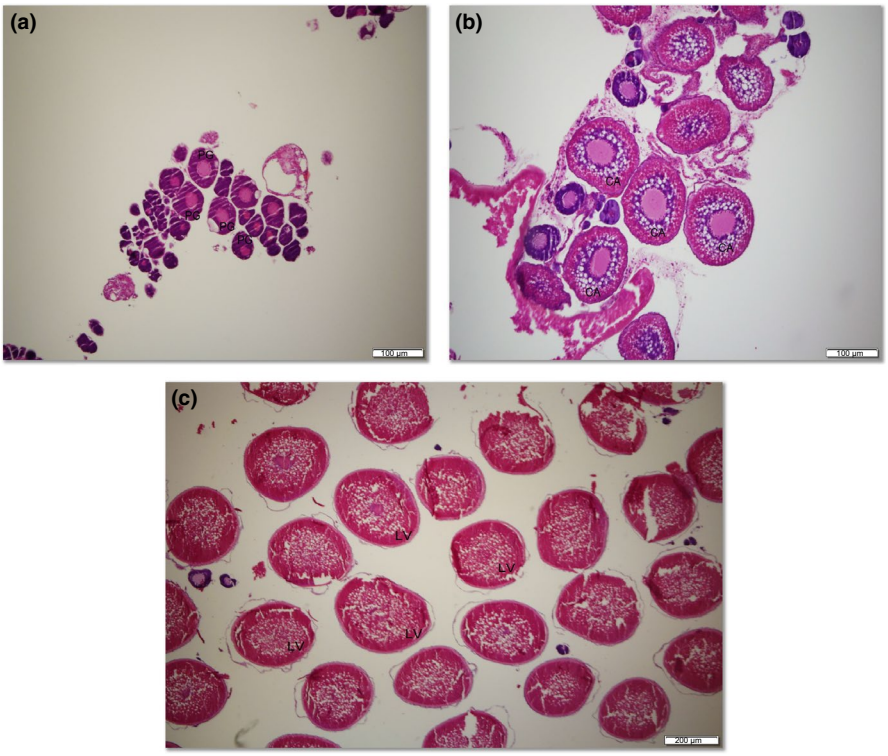

FIGURE 1 Micrographs of histologically sectioned oocytes collected in March 2018 from female bonefish (*Albula* spp.) reared in the laboratory at Cape Eleuthera Institute, Eleuthera, The Bahamas under ambient conditions. Three different developmental oocyte stages are present: (a) primary growth (PG), (b) cortical alveolar (CA) and (c) late vitellogenic (LV)

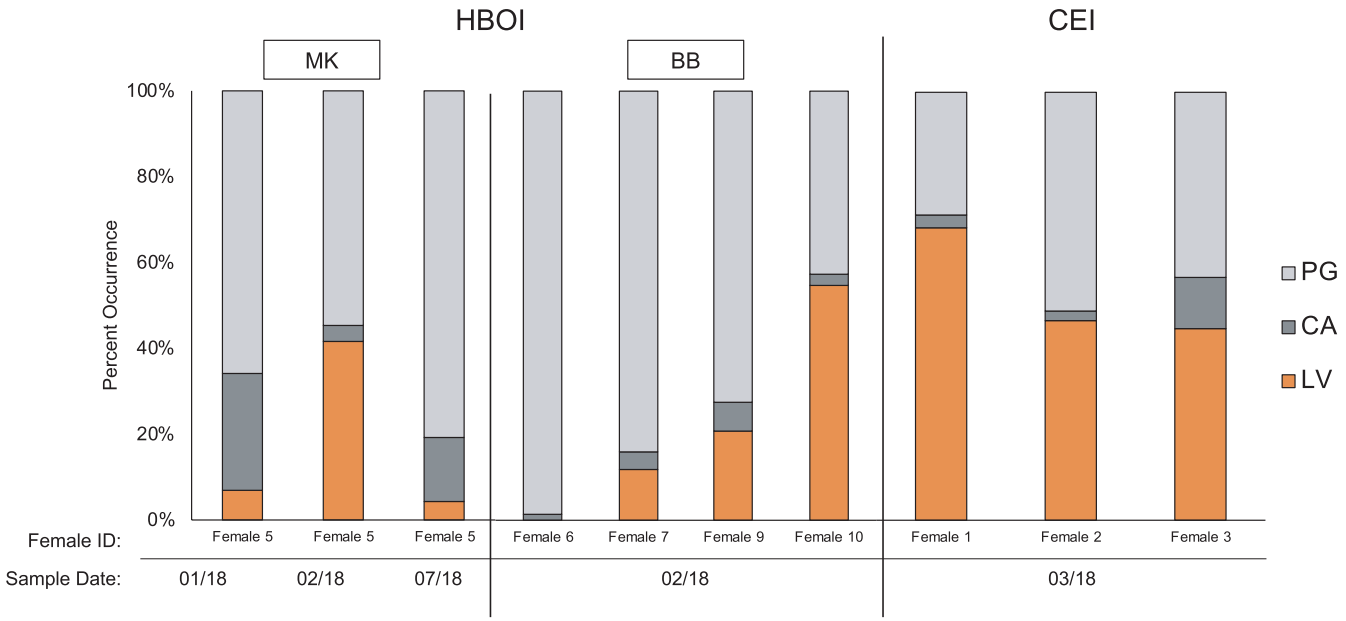

FIGURE 2 Proportional occurrence for each of the three oocyte stages: primary growth (PG), cortical alveolar (CA) and late vitellogenic (LV) found in captive bonefish females (*Albula* spp.) sampled at both Harbor Branch Oceanographic Institute (HBOI), Fort Pierce, FL, USA and Cape Eleuthera Institute (CEI), Eleuthera, The Bahamas. Females held at HBOI were captured from two separate locations (Middle Keys [MK] and Biscayne Bay [BB]) and reared under simulated photothermal

or HD (n = 2; Table 1). Females 4 and 5 received the same dose of CPE injections (LD) in 12/2017, 01/2018 and 02/2018 (n = 2). After short cycling, the same females received HD (n = 2; Table 1). Females 6, 7, 8, 9 and 10 did not receive any CPE injections before 02/2018, then received VLD in 02/2018 (n = 3) followed by HD twice (n = 2; Table 1).

Wild adult bonefish from The Bahamas (*Albula* spp.) were collected with a seine net from the waters surrounding Eleuthera (n = 44) in April 2017. Fish were kept in tanks with flow-through seawater (four tanks of 13,000 L [n = 11/tank]) and were exposed to ambient photoperiod and temperature for 12 months. Fish were palpated, cannulated and blood samples were taken (n = 11) monthly on a rotating

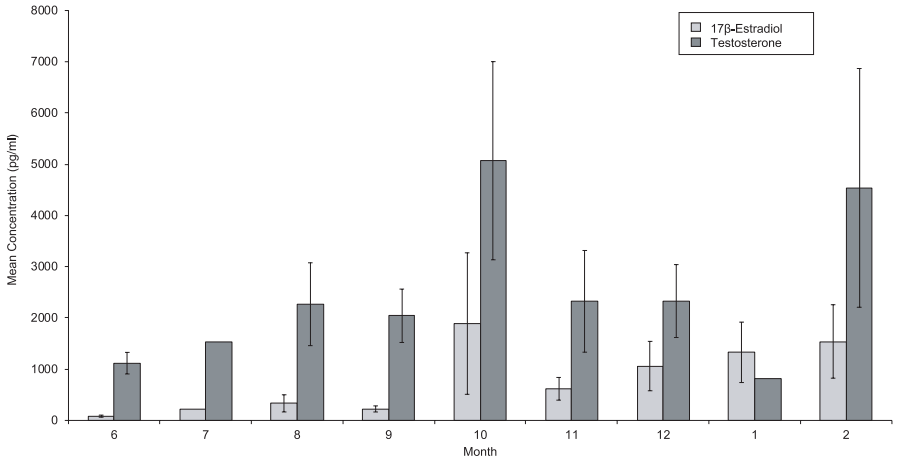

FIGURE 3 Plasma concentrations of 17β-estradiol and testosterone (mean ± *SEM*) from female bonefish (*Albula* spp.) reared in captivity at Cape Eleuthera Institute, Eleuthera, The Bahamas under ambient conditions

TABLE 3 Fatty acid composition (mean ± *SD*, expressed as percentage of total neutral and polar lipids detected) in captive bonefish (*Albula vulpes*) oocytes from Harbor Branch Oceanographic Institute (HBOI), Florida, USA and Cape Eleuthera Institute (CEI), Eleuthera, The Bahamas

	Neutral		Polar	
Fatty acids	HBOI	CEI	HBOI	CEI
14:0	4.49 ± 0.46	3.90 ± 0.83	1.56 ± 0.85	0.78 ± 0.07
16:0	24.38 ± 9.20	19.91 ± 1.82	29.48 ± 3.75	29.31 ± 0.55
18:0	5.78 ± 1.03	7.04 ± 0.26	12.07 ± 3.70	13.28 ± 0.49
Total SFA[a]	38.09 ± 10.26	32.93 ± 2.72	44.08 ± 6.96	44.72 ± 1.15
16:1 n-7	12.69 ± 0.05	11.86 ± 2.27	4.77 ± 1.47	3.56 ± 1.18
18:1 n-9	27.85 ± 4.48	29.84 ± 3.24	14.06 ± 0.90	10.82 ± 0.99
18:1 n-7	5.57 ± 1.45	6.45 ± 0.25	3.50 ± 0.64	4.07 ± 0.74
Total MUFA[b]	48.96 ± 6.31	52.65 ± 5.66	23.09 ± 1.93	19.55 ± 1.53
18:2 n-6	1.05 ± 0.31	0.99 ± 0.06	–	–
20:4 n-6 (ARA)	0.99 ± 0.04	1.12 ± 0.41	5.42 ± 1.24	4.74 ± 0.72
20:5 n-3 (EPA)	2.80 ± 0.35	2.03 ± 0.93	6.34 ± 0.51	4.52 ± 0.27
22:5 n-3 (DPA)	1.17 ± 0.17	0.76 ± 0.38	2.10 ± 0.23	1.58 ± 0.10
22:6 n-3 (DHA)	5.10 ± 2.39	8.03 ± 4.49	16.84 ± 6.38	21.86 ± 0.46
Total PUFA[c]	12.86 ± 3.82	13.19 ± 6.64	32.14 ± 7.92	35.47 ± 0.23

Abbreviations: –, not detecte; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; SFA, Saturated fatty acids.
[a]Includes 11:0, 12:0, 13:0, 15:0, 17:0, 20:0, 22:0 whose combined percentages are ≤0.5% of total neutral or polar fatty acids.
[b]Includes 14:1 n-5, 14:1 n-7, 15:1, 17:1, 20:1, 24:1 n-9, whose combined percentages are ≤0.5% of total neutral or polar fatty acids.
[c]Includes 18:3 n-6, 18:4 n-3, 20:3 n-6, 22:5 n-6, whose combined percentages are ≤0.5% of total fatty acids.

sampling regime. No hormone trials were conducted on these fish (Table 2). Data from identified females only (n = 4) are presented here.

Oocytes stored in 10% neutral buffered formalin during collection from females were prepared following Barber (1996) and Wilson et al. (2005). For each subset of oocytes (n ≥ 40) from histology slides, developmental stages were categorized as vitellogenic (Vtg), cortical alveolus (CA) and primary growth (PG; Crabtree, Snodgrass, & Harnden, 1997) (Figure 1). Estradiol (E2) and testosterone (T) concentration levels were quantified via enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical Company) and were analysed via absorbance (405 nm wavelength) using a microplate reader (Biotek, Synergy H1). Lipids from a pool of oocytes were extracted following Parrish (1999). All fatty acid methyl esters (FAME) were analysed using the MIDI Sherlock® Microbial Identification System (MIS). Free amino acids were extracted from oocytes and were analysed by Protein Chemistry Laboratory. Monthly comparisons of both E2 and T plasma concentrations were conducted using a one-way ANOVA with month as a fixed factor. Analysis was conducted using

TABLE 4 Free amino acid composition (percent of total free amino acids; mean ± *SD*) in captive bonefish (*Albula vulpes*) oocytes from Harbor Branch Oceanographic Institute (HBOI), Florida, USA

Essential free amino acids	
Histidine (HIS)	4.49 ± 0.02
Isoleucine (ILE)	3.43 ± 0.01
Leucine (LEU)	6.61 ± 0.03
Lysine (LYS)	4.52 ± 0.19
Methionine (MET)	1.18 ± 0.03
Phenylalanine (PHE)	2.31 ± 0.03
Threonine (THR)	5.26 ± 0.01
Valine (VAL)	4.48 ± 0.01
Conditionally essential free amino acids	
Arginine (ARG)	3.45 ± 0.04
Proline (PRO)	4.67 ± 0.06
Tyrosine (TYR)	2.82 ± 0.16
Non-essential free amino acids	
Alanine (ALA)	12.49 ± 0.05
ASX[a]	8.03 ± 0.04
GLX[b]	10.34 ± 0.16
Glycine (GLY)	9.80 ± 0.17
Serine (SER)	7.06 ± 0.06
Taurine (TAUR)	9.05 ± 0.14
Total free amino acid (g/g wet mass)	0.36 ± 0.001

[a]Includes Aspartic acid (ASP) and Asparagine (ASN).
[b]Includes Glutamine (GLN) and Glutamic acid (GLU).

R software (R Core Team 2014). Permutational analysis of variance (PERMANOVA with 9,999 permutations) was performed on fatty acid profiles.

Overall, no ovulation or spawning occurred; however, gonad development was further induced via CPE injection. One female (number 5) developed eggs that ranged from 250 µm in 12/2018 to 400 µm in 02/2018 after three similar LD's of CPE. Moreover, Vtg stage increased from 8% in 01/2018 to 42% in 02/2018 of total stages (Figure 2). Oocytes size was 375 µm after the same female was exposed to a short cycle, and received HD of CPE. Females from BB (6, 7 and 9) developed oocytes ranging from 100–250 µm, with 98%, 84% and 71% of oocytes at PG stage, respectively (Figure 2). Female 10 developed the largest oocytes (500 µm) with Vtg stage being the most prominent (>50%). The mean ± *SE* vitellogenic oocyte diameter of samples collected from CEI fish was 473 ± 58 µm. Vitellogenic stage was the most abundant from female number 1 (>68% of total stages), which successfully developed oocytes for four consecutive months (Figure 2 and Table 2). Females number 2 and 3 had PG or CA stages as the most abundant (Figure 2). There were no significant differences in E2 and T values between months (E2: $F_{(1, 35)} = 0.345$, $p = 0.561$; T: $F_{(1, 26)} = 0.182$, $p = 0.673$; Figure 3). Oocytes from both CEI and HBOI, had similar fatty acid composition ($p > 0.05$; Table 3). The three essential FAs; arachidonic acid (ARA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were two times higher in the lipid polar fraction. Eight essential FAAs were identified in bonefish oocytes (Table 4). All non-essential FAA relative percentages were higher than 7% of total FAAs (Table 4).

Captive rearing of adult bonefish under ambient conditions resulted in significant gonad development, but was unsuccessful for ovulation or spawning. Therefore, results suggest that bonefish either require a longer time to adapt to captivity before they respond to environmental spawning cues or perhaps behavioural group dynamics are needed to initiate latter stages of maturation and spawning. Reports from Atlantis Aquarium Resort, The Bahamas, indicated that bonefish (n ~ 250) were in tanks for multiple years before they spawned under ambient conditions (Wert, personal communication).

Carp pituitary extract injections did not induce spawning, but helped to stimulate the first stages of gonad development in captive bonefish. Oocytes of Japanese eels (*Anguilla japonica*) in captivity are only considered mature when they reach sizes >700 µm (Kagawa, Tanaka, Ohta, Okuzawa, & Hirose, 1995). In our study, only one female had oocytes large enough to be considered mature, while the oocytes of the other fish sampled were smaller and either at early vitellogenic or primary growth stages. Given the close lineage of bonefish to eels within the superorder Elopomorpha, it was assumed that CPE would also be effective for advancing bonefish maturation, however, this was not the case.

Generally, estradiol values increase dramatically in bonefish during spawning season, as females undergo vitellogenesis. Values during this time vary dramatically by individual but are typically much higher (~4,000 pg/ml) than what is observed during non-spawning months (<1,000 pg/ml; Luck et al., 2018). In captive fish, estradiol values were substantially lower than what were commonly observed in wild bonefish captured during their spawning migration to deep water, but similar to fish captured on the flats, suggesting that some fish may be able to undergo vitellogenesis in the near future.

Bonefish at HBOI and CEI were fed diets that closely resembled those of wild bonefish (Crabtree, Stevens, Snodgrass, & Stengard, 1998; Murchie, 2010; Stein et al., 2012). However, we found that neutral lipid PUFAs levels were two times lower in oocytes from the captive fish than those collected from wild fish (Mejri et al., 2018), suggesting potential deficiencies in ARA and EPA levels, but not DHA. Therefore, it is possible that the lack of these essential fatty acids in the captive fish diet could have negatively impacted gonad maturation, as observed in other fish species (Izquierdo, Fernández-Palacios, & Tacon, 2001).

Free amino acid profiles were dominated by neutral amino acids such as leucine, valine, isoleucine, alanine and serine. These findings are in line with earlier observations of wild bonefish oocytes (Mejri et al., unpublished data) and for 23 species of tropical marine fish eggs (Rønnestad, Robertson, & Fyhn, 1996). Our data suggest that the FAA pool in bonefish oocytes may not only be critical for the final oocyte maturation and hydration, but also used for energy purposes during embryogenesis.

In summary, photothermal manipulations and hormone injections were not effective for bonefish. However, this novel work has significantly advanced research in this realm and is important for the understanding of bonefish reproductive biology. Future research should establish if seasonal variations in salinity, temperature and moon

phase exist where wild bonefish spawn and if so, does it serve as an environmental cue for gonad development and spawning in bonefish. Moreover, CPE injections can continue to be used, but other hormones that have been used successfully to spawn other fish in captivity should be attempted to determine a more effective protocol to induce gonad maturation.

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ETHICAL APPROVAL

The experimental protocol received approval from the Florida Atlantic University's Institutional Animal Care and use Committee (IACUC, protocol A16-34).

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BRIEF COMMUNICATION

Induced spawning and embryonic and early larval development of bonefish (*Albula vulpes*)

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Abstract

Bonefish (*Albula vulpes* L.) are a highly prized sport fish. Despite their economic importance, populations in the Florida Keys and Caribbean are in decline, with the early life history undescribed. Injections of carp pituitary extract into *A. vulpes* during the advanced stages of ovarian development induced ovulation and spawning. Embryos were sampled hourly until hatching into undeveloped, yolk-sac leptocephalus larvae. These larvae survived 56 h post-hatch, when myomeres and eyes were developing but not the mouth. These results inform future research on the reproduction and early life history of *A. vulpes*.

KEYWORDS

bonefish, CPE, embryos, leptocephalus larvae, ovulation, spawning

Understanding the reproductive biology of teleost fishes provides critical knowledge of their population biology, behaviour, habitat associations and other biological and ecological characteristics needed for the development of management and conservation strategies. Studies of reproductive processes in natural systems are often supplemented by spawning and larval rearing in laboratory settings. Although many marine fish species have been studied extensively, the analysis of the superorder Elopomorpha has been hindered by the difficulty in collecting reproductive specimens and inducing spawning in laboratory culture.

Bonefishes (*Albula* spp.) comprise at least 10 morphologically similar species that are found globally in coastal waters of the tropics and subtropics (Colborn *et al*., 2001; Pfeiler *et al*., 2008) and support economically important recreational and subsistence fisheries throughout their ranges (Adams *et al*., 2012). Four of these species are found in the Caribbean Sea, and subtropical western North Atlantic Ocean, with *Albula vulpes* supporting recreational fisheries with an estimated annual value of US$465 million in the Florida Keys (Fedler, 2013), US $161 million in The Bahamas (Fedler, 2019), and US$50 million in Belize (Fedler, 2014). In other locations, *A. vulpes* is an important component of an artisanal harvest fishery [*e.g*., Cuba (Rennert *et al*., 2019)]. Despite the economic and cultural importance, reproductive and early life-history stages of *A. vulpes* have not been studied (Adams *et al*., 2014). In recent decades, the population of *A. vulpes* has declined in the Florida Keys (Rehage *et al*., 2019) and in numerous locations in the Caribbean (Adams *et al*., 2012; Santos *et al*., 2017), leading to the listing of this species as "near-threatened" by the IUCN (Adams *et al*., 2012).

Adult *A. vulpes* typically have a high site fidelity for their foraging habitats on shallow (<1 m) flats (Boucek *et al*., 2019). Before full and new moons from November to April, adults migrate up to 100 km, forming large prespawning aggregations near the edge of deep-water drop-offs with depths >1000 m (Adams *et al*., 2019; Crabtree *et al*., 1997; Danylchuk *et al*., 2011; Danylchuk *et al*., 2019). Prespawning aggregations are estimated at about 10,000–15,000 fish. During prespawning hours in the late afternoon, bonefish engage in porpoising behaviour at the surface, apparently to ingest air into their swimbladders and intestines. At dusk, the aggregation moves offshore, where spawning occurs: total water depth exceeds 1000 m, and bonefish descend to 50–60 m for several hours before rapidly rising towards the surface and apparently releasing their gametes (Danylchuk *et al*., 2011). Little is known about the biology and ecology of the pelagic leptocephali. Leptocephalus larvae reside offshore for 41–71 days before metamorphosis and recruitment to inshore

*These authors should be considered as joint first authors.

[Correction added on 25 March 2020, after first online publication. The name of the seventh author, Jonathan M. Shenker, appeared incorrectly as "Shenker M. Jonathan" in the original online publication and in the print version of the article. This has now been amended in the online version.]

habitats (Mojica *et al*., 1995), where larvae metamorphose into juveniles in protected sandy and muddy bays (Haak *et al*., 2019).

Initial attempts to induce spawning in bonefish focused on the development of procedures to assess reproductive conditions in females before spawning and identification of candidate hormones responsible for triggering the final stages of oocyte development (Mejri *et al*., 2019). Mejri *et al*. (2019) demonstrated that the advancement of gonad development is possible in the laboratory using a combination of environmental manipulation and carp pituitary extract (CPE) injections, when oocytes are ≤700 μm, but no ovulation or spawning occurred (Mejri *et al*., 2019). The first, successful induced spawning of *A. vulpes* is reported here. The specific objectives of this study were to (a) capture reproductively mature fish from prespawning aggregations, (b) induce final oocyte maturation and ovulation through administration of CPE and (c) describe and document embryonic and early larval development.

Sexually mature *A. vulpes* (n = 6 females; n = 12 males) were collected using hook and line in December 2017 and November 2018 in prespawning aggregations that were discovered in Eleuthera and Great Abaco, The Bahamas, respectively (Adams *et al*., 2019). Precise locations are not published in the interest of aggregation conservation.

Broodstock collected in Eleuthera [n = 2 females; n = 4 males; fish designation = CEI; fork length (FL) = 320–390 mm] and near Great Abaco (n = 4 females; n = 8 males; fish designation = ALB; FL = 405–480 mm) were placed in 1500 l circular tanks under ambient light and supplied with flow-through sea water. The criterion for selecting females for spawning trials was cannulated oocytes >700 μm diameter (Kagawa *et al*., 1995; Mejri *et al*., 2019). Freely flowing milt was used to select males.

Five females received initial intraperitoneal injections of 20 mg kg^{-1} of CPE (Stoller Fisheries, Spirit Lake, IA, USA) in the early evening within 8–10 h after capture. Another CPE injection (20 mg kg^{-1}) was administrated 24 h later. ALB-2 received only a single injection of CPE (60 mg kg^{-1}) to test the effect of a larger, single dose on the final oocyte maturation and ovulation. Fish were anaesthetised before they received injections. All males released sperm because of abdominal pressure, so no hormonal injections were administrated. All fish were monitored at 4–6 h intervals until signs of ovulation (a significantly distended abdomen) occurred. For spawning, the fish were anaesthetised, and eggs and sperm were extruded into separate bowls by gentle massage of the abdomen. Sperm was then added to the eggs and gently mixed with a feather. Ambient sea water was then added to activate sperm and promote fertilisation. Fertilised eggs were left undisturbed for 5 min and then rinsed three times with ambient sea water. Fish were returned to their tanks after spawning.

Fertilised eggs were incubated in two 6 l polycarbonate containers supplied with filtered, ambient sea water, which were placed in a static 100 l water-bath to maintain temperature (24.8 ± 1°C). A small air stone in each container provided gentle aeration.

Partial water changes (25%) were conducted every hour, with a 50% water change every 6 h during embryogenesis. After hatching, 50% water changes were conducted every 4 h, and dead eggs and larvae were removed from the bottom of the incubation container to maintain water quality.

The study was performed with permission from the Bahamas Department of Marine Resources (permit number MAMR/FIS/17) and received ethical approval from the Florida Atlantic University's Institutional Animal Care and Use Committee (protocol A16-34).

Photographs of cannulated oocytes and developing embryos were taken every hour from fertilisation through hatching, using a 1.3 MP Dino-Lite Edge microscope (Dino-Lite, Torrance, CA, USA). Larvae were photographed initially at 2 h intervals [hatching, *i.e*., 0, 2, 4 and 6 h post-hatch (hph)], and then sampling was reduced to every 4 h until no live larvae remained.

Females captured at the prespawning aggregation sites contained oocytes at advanced stages of development and completed their maturation in response to CPE administration. Oocytes removed from the ovaries within hours after capture but prior to CPE injection had a uniformly smooth, opaque, orange-yellow appearance (Figure 1a). Almost all oocytes were in the late vitellogenic stage of development (Figure 1b,c) with mean diameter 713–840 μm (Table 1). Oocytes collected after 24 h, after the first CPE injection, had increased in size to about 900 μm (Table 1), 76%–89% underwent germinal vesicle (nucleus) migration (GVM) towards the periphery of the oocyte (Figure 1d) and 6%–11% had begun breakdown of the germinal vesicle membrane (germinal vesicle breakdown). By 12 h after the second injection, CEI-1 had a distended abdomen, ovulation had occurred and fully hydrated eggs were easily released with light abdominal massage. Eggs increased to a mean diameter of 1.1 mm, with the contents separating into transparent yolk proteins and numerous oil droplets (Figure 1e). CEI-2, ALB-3 and ALB-4 were not artificially spawned but were found to have released eggs in the tank overnight and were unrecoverable. Interestingly, CEI-1, CEI-2, ALB-1, ALB-3 and ALB-4, which received two intraperitoneal CPE injections (20 mg kg^{-1}) spaced 24 h apart, and ALB-2, which is the only fish to receive a single dose of CPE (60 mg kg^{-1}), all resulted in oocytes completing final maturation, hydration and ovulation (Table 1).

Eggs before and immediately after fertilisation had a mean diameter of 1.2 ± 0.47 mm, with the yolk having a mean diameter of 1.05 ± 0.23 mm. The dense array of oil droplets on the upper surface of the egg obscured the visual examination of early embryonic development, but a disc of cells could be seen on the lateral surface of the egg by around 8 h post-fertilisation (hpf). Oil droplet coalescence did not begin until 21 hpf and was not complete for several embryos before hatching, which occurred at 25–26 hpf. The zygote and cleavage stages lasted 1–2 h, with blastula (Figure 1f), gastrula and segmentation stages (Figure 1g) each persisting for 6–9 h. The pharyngula stage (Figure 1h) occurred during the final 2–4 h before hatching.

After 25–26 h of development, embryos hatched into morphologically primitive larvae (Figure 1i), with a mean length of 3.36 mm. Mouth or digestive system was not present, and eyes were just beginning to develop. The remnants of the yolk extended nearly the full length of each larva, with oil droplets having coalesced into a single elongated droplet at the anterior of the yolk (Figure 1j,k). Larvae grew at an average rate of 1.7 mm NL day^{-1} to a mean length of 7.4 mm

TABLE 1 Summary of oocyte development of *Albula vulpes* females from Eleuthera (CEI-1 and CEI-2) and Abacos (ALB-1, ALB-2, ALB3 and ALB-4) islands, The Bahamas, showing oocyte diameter (±S.D.) and relative percentages of oocytes in different developmental stages (DS) at initial sampling (T_0), 24 h (T_{24}) and time strip spawned (T_X) hours following CPE injections. ALB-2 received only a single injection of CPE (60 mg kg^{-1}), whereas the other five received two intraperitoneal CPE injections (20 mg kg^{-1}) spaced 24 h apart

	Oocyte diameter (μm)			Relative percentage of DS						
	Hours post CPE injection			T_0			T_{24}			
Fish ID	T_0	T_{24}	T_X	LV	GVM	GVBD	LV	GVM	GVBD	Time spawned
CEI-1	840.0 ± 15.7	902 ± 41.8	1049.3 ± 97.7	98	2	0	13	76	11	48 h post T_0 – strip spawn
CEI-2	713.6 ± 45.9	928.5 ± 34.6	–	100	0	0	20	74	6	≥28 h post T_0 – eggs released into tank
ALB-1	701.1 ± 50.8	926.7 ± 42.1	980.1 ± 97.4 measure at ~33 post T_0	76	16	8	28	62	10	33 h 30 min post T_0– strip spawn
ALB-2	988.5 ± 50.8	–	–	0	61	39	–	–	–	12 h post T_0 – strip spawn
ALB-3	938.2 ± 65.5	1012.3 ± 12.8	–	0	90	10	0	0	100	≥28 h post T_0 – released eggs into tank
ALB-4	704.9 ± 46.7	961.5 ± 41.45	–	80	20	0	33	63	3	≥28 h post T_0 – released eggs into tank

Note: Symbol (–) in indicates that no data were collected or the absence of sampling at that time. Oocyte diameter measure represents the mean ± S.D. from 35 to 37 oocytes.
Abbreviations: GVBD, germinal vesicle breakdown; GVM, germinal vesicle migration; LV, late vitellogenic stage.

NL at 52 hph. Larvae nearly completed the full utilisation of their yolk reserves by 56 hph, when the final larvae died. Larvae initially oriented vertically in rearing containers, with heads oriented towards the surface, and generally remained motionless, although occasional sporadic darting movements were observed.

The reproductive behaviour of bonefish provides unique challenges for the analysis of their spawning biology and early larval biology in wild populations and for artificial propagation in laboratory settings. The injection of CPE into bonefish collected from prespawning aggregations succeeded in stimulating final oocyte maturation and ovulation, even without the gulping and deep-diving behaviour observed previously in wild bonefish during spawning.

The only other Elopomorph fishes that have been successfully spawned in captivity are the Japanese eel *Anguilla japonica* (Ohta *et al*., 1997; Tanaka *et al*., 2001; Temminck & Schlegel, 1846); European eel *Anguilla anguilla* (Palstra *et al*., 2005; Pedersen, 2003); and two species of New Zealand eels, *Anguilla diefenbachii* and *Anguilla australis* (Lokman & Young, 2000). Although eels and bonefish share an evolutionary heritage, they have different spawning behaviours, and yet they still present common challenges to reproduction under controlled conditions. Eels reportedly spawn at night in the shallower depths of their migratory zone (Schabetsberger *et al*., 2016). Shallow-water-dwelling bonefish appear to gulp air into their physostomous swimbladders during the porpoising behaviour (Danylchuk *et al*., 2011; Danylchuk *et al*., 2019). The turgid swimbladder of one female bonefish (FL = 320 mm) was nearly cylindrical, with a volume of 183 cc. At a depth of 50 m, the swimbladder collapsed, leaving 177 cc of "empty" abdominal space to be filled by hydrating eggs. For bonefish, and possibly eels, swimbladder expansion during vertical spawning rushes may also assist with gamete release during vertical spawning rushes exhibited by snappers, groupers and many other marine species (*e.g*., Colin, 1992; Domeier & Colin, 1997).

The logistical impossibility of replicating long-distance migrations and vertical spawning rushes in culture systems may be, at least, partially overcome by the administration of reproductive hormones to induce oocyte development. Studies on captive *A. anguilla* and *A. japonica* determined that weekly injections (20 mg kg^{-1}) of carp or salmon pituitary extracts for 10–25 weeks stimulate gonadal development in the laboratory (*e.g*., Ohta *et al*., 1997; Palstra *et al*., 2005). Gametes were typically strip spawned for controlled fertilisation, although Van Ginneken *et al*. (2005) reported volitional spawning of eels after hormone injections.

Vitellogenesis and oocyte maturation of bonefish used in this study occurred primarily on their home territories and during the migration towards the prespawning aggregation site (Luck *et al*., 2019). The difficulty in capturing and retaining fish in culture tanks was successfully counteracted by the administration of two 20 mg kg^{-1} doses or a single dose of 60 mg kg^{-1} of CPE to fish that had oocytes ≥700 μm when initially examined. The sequence of GVM and breakdown, separation of lipids and yolk proteins, hydration and ovulation, however, took 25–36 h after CPE administration, which is slower than that in wild fish which takes approximately 12 h or less, based on eggs collected from fish before and after spawning (Mejri *et al*., 2019). In a previous study, smaller oocytes (<700 μm), either at early vitellogenic or primary growth stages, did not complete maturation after CPE was injected (Mejri *et al*., 2019).

Embryonic development of *A. vulpes* was rapid, with hatching occurring 25–26 hpf. This development rate was similar to that observed in several anguillid eels (Ahn *et al*., 2012; Sørensen *et al*., 2016). In comparison to pelagic eggs of other marine fish species, hatch time was similar, especially for those species reared at an equivalent water temperature to our study (Pauly & Pullin, 1988).

Initial larval growth rates in this study were similar to leptocephali of eel species, although variation between tropical and temperate eels has been found (Miller, 2009). Although the larval survival of *A. vulpes*

was not long enough to effectively compare oil utilisation, growth rates would have likely declined as lipids provided the remaining nutrients to support the formation of the mouth and jaw. Another function that the oil droplet serves is that it aids in buoyancy (Mejri *et al*., 2018), as observed in other fish species (Sørensen *et al*., 2016; Williams *et al*., 2004), until larvae become more effective swimmers.

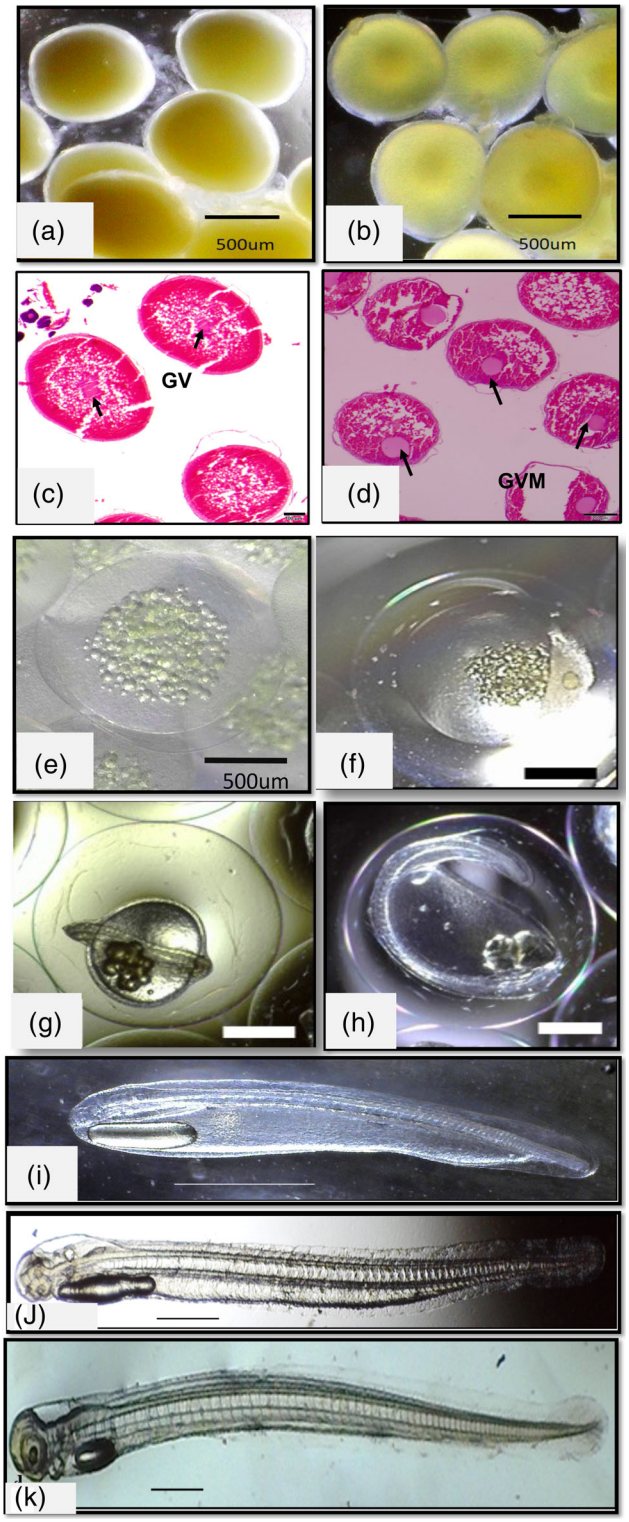

FIGURE 1 Legend on next column.

While this study demonstrates that oocytes can be obtained through hormone administration in *A. vulpes* and produce viable offspring, further refinement of the procedure is required. For future spawning attempts, it is recommended that egg diameters be 800–900 μm, the germinal vesicle be at or near the animal pole and oil droplets be transparent before the intraperitoneal injection of CPE to induce spawning. This work provided a novel baseline for understanding the early development of *Albula* spp. and will guide future research that aims to understand reproductive behaviours, prespawning and spawning site selection, larval behaviour, growth and oceanic larval transport. Future research efforts should focus on whether delayed oil droplet coalescence, observed in this study, is typical of the development of elopomorph oocytes or a consequence of captive spawning. Furthermore, progress in the rearing of larvae to exogenous feeding stages is needed before the viability of an artificial propagation programme for *A. vulpes* can be assessed.

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AUTHOR CONTRIBUTIONS

W.R.H. helped with broodstock collection; development of spawning and rearing protocols; hormone injections; and light microscopy, measurements, descriptions and analysis and manuscript preparation. S.M. helped with ideas; data generation; data analysis; histology preparation and analysis; and manuscript preparation. A.C.C. aided in collection and maintenance of broodstock, implementation and development of hormone injection, strip spawning and larval rearing

FIGURE 1 Images and micrographs of histologically sectioned oocytes, embryonic development and larvae of *Albula vulpes*. (a) Smooth, opaque, orange-yellow appearance of an oocyte; (b) and (c) germinal vesicle (GV) central position in oocytes before the first carp pituitary extract (CPE) intraperitoneal injection; (d) germinal vesicle migration (GVM) in oocytes 24 h post CPE injection; (e) a hydrated oocyte ready to be fertilised, with the contents separating into transparent yolk proteins and numerous oil droplets; (f) embryo in the blastula stage (8 h post-fertilisation); (g) embryo in the segmentation stage (21 h post-fertilisation); (h) embryo in the pharyngula stage (24 h post-fertilisation); (i) larvae at 2 h post-hatch (hph); (j) larvae at 30 hph; and (k) larvae at 52 hph. Scale bar for images f–h = 0.5 mm and for i–k = 1 mm. Sub-samples of oocytes of a, b, e, f and g–k were stored in 10% neutral buffered formalin until examination under the microscope, whereas those of oocytes of c and d were stored in 10% neutral buffered formalin and subsequently transferred into 70% ethanol before histological preparation; oocytes were dehydrated through a series of ethanol solutions (70%–100%) for 60 min, clarified in toluene and embedded within paraffin wax

procedures and also in editing manuscript. P.S.W. helped with ideas, data analysis, manuscript preparation and funding. T.E.V.L. helped with collection and maintenance of broodstock and with implementation and development of hormone injection and initial studies on oocyte maturation in wild bonefish and development of hormonal procedures to induce final oocyte maturation and ovulation; A.J.A. helped with ideas, manuscript preparation and funding; J.M.S. aided in initial studies on oocyte maturation in wild bonefish and development of hormonal procedures to induce final oocyte maturation and ovulation and also in supervision of hormonal induction, embryological and early larval development reported in this paper and manuscript preparation.

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ORIGINAL ARTICLE

Lipid Composition and Utilization in Early Stage Leptocephalus Larvae of Bonefish (*Albula vulpes*)

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Abstract In order to encourage the survival of both captive and wild populations of bonefish (*Albula vulpes*), a deeper understanding of the species' early developmental characteristics is necessary. During ontogenesis, bonefish utilize lipids as a source of energy before the start of exogenous feeding. The goal of this study is to gain insight into the energetic needs of bonefish leptocephalus larvae in the post-hatch larval stage. Broodstock were collected in the wild and hormone induced. Spawning yielded eggs that were fertilized and were then incubated until hatching. Larval development was monitored throughout the duration of the trial until all larvae perished. Samples of larval tissue were taken to the lab for lipid analysis and composition was compared at different developmental stages. Larval lipid composition was significantly different across sample groups showing a change in lipid content related to development. After hatching, larvae gradually depleted wax esters-sterol ester (WE-SE) reserves over a period of 4 days, while simultaneously increasing hydrocarbon (HC). The role of WE-SE is seemingly tied to both buoyancy and energy reserves due to its high abundance immediately post-hatch and selective catabolism. As larvae weaned off of the nutrition provided by the yolk, exogenous feeding began to diversify lipid composition and overall lipid reserves were depleted. Future directions included the development of optimal larval feeds based on this analysis in order to gain more insight into the nutritional needs and requirements during the critical leptocephalus stages.

Keywords Bonefish · Larval nutrition · Leptocephalus · Lipid classes · Ontogeny

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Abbreviations

AMPL	acetone-mobile polar lipids
D_o	oil droplet size
D_Y	yolk sac size
FFA	free fatty acids
HC	hydrocarbons
hph	hours post hatch
KET	ketones
PL	phospholipids
ST	sterols
TAG	triacylglycerols
TL	total length
TLS	total lipids
WE-SE	wax esters-steryl esters
WW	wet weight

Introduction

Early development of the unique leptocephalus larvae of elopomorph fishes (eels, spiny eels, bonefish, tarpon, and

Supporting information Additional supporting information may be found online in the Supporting Information section at the end of the article.

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ladyfish) leads to larvae that are morphologically distinct from other teleost larvae in terms of feeding modalities and growth patterns. One of the most economically important groups of Elopomorphs are a suite of morphologically similar species of bonefishes (Albuliformes) that support recreational and consumptive fisheries in tropical and semitropical shallow water habitats around the world. The focus of this paper is *Albula vulpes*, hereafter referred to as bonefish. Bonefish are the dominant fishery species in southern Florida, the Bahamas, Cuba, and other regions throughout the Caribbean Sea. Currently, *A. vulpes* is listed as a "near threatened" by the International Union for Conservation of Nature (IUCN), which highlights the urgency for this type of research (Adams et al., 2012). Studies regarding bonefish development provide insight into better understanding of the reproductive biology of wild populations and have implications for developing protocols to culture bonefish and other Elopomorphs in captivity.

Previous studies on reproductive biology of wild bonefish have characterized how temporal and spatial differences influence oocyte development and production of reproductive hormones (Luck et al., 2019; Mejri et al., 2019a; Mejri et al., 2020; Santos et al., 2017). Other studies have identified pre-spawning aggregation locations and behaviors (Adams et al., 2019; Danylchuk et al., 2011, 2019), characterized spawning activity (Lombardo et al., n. d.), explored the use of hormones to induce spawning in wild-caught fish, and provided an initial description of embryological development (Halstead, 2019). This study regarding the lipid composition of the earliest leptocephalus larval stages of bonefish begins to fill the gap in understanding the suite of processes that affect reproduction and recruitment of this valuable fishery species.

The long, ribbon-shaped leptocephali have a body composition and feeding behaviors that are different from other teleost larvae. Unlike most larval fishes, which are visual feeders with a diet of plankton, leptocephali appear to have a diet based on mucilaginous "marine snow" derived from a wide array of sources including discarded mucus houses of appendicularians and other gelatinous materials (Chow et al., 2019; Miller et al., 2020). At hatching, leptocephali are morphologically primitive larvae that have not yet developed jaws or digestive tracts, eyes, and other characteristics necessary for location and consumption of food (Halstead, 2019). Early development to the onset of exogenous feeding must be fueled by nutrients transferred from the mother to the eggs prior to spawning and remaining after embryonic development. Until recently, the only elopomorph fishes successfully bred in captivity that would enable analysis of the impact of broodstock nutrition on larval development are several species of anguilliform eels (Mochioka et al., 1993; Tanaka, 2015; Tanaka et al., 2003). Newly developed methods to induce spawning in wild-caught bonefish (Halstead et al., 2020; Mejri et al., 2019a; Mejri et al., 2020) offer the opportunity to describe the nutritional composition of early larvae, providing a basis for production and growth of larvae under controlled conditions.

Although there are no previous data on the lipid composition of larval bonefish, it is well known that lipids provide an energy source for newly hatched larvae of other teleost species and that the quantification of specific lipid classes can give clues as to the nutritional needs of larvae in early development (Rainuzzo et al., 1997). Lipid reserves play a role in metabolic processes as well as in cellular membrane biogenesis. Previous studies conducted by our group have shown that mature bonefish oocytes are lipid-rich consisting of about 20% lipids of total wet weight, dominated by wax esters and steryl esters (WE-SE) (Mejri et al., 2019b, 2020). This paper characterizes for the first time the lipid composition of bonefish leptocephalus larvae during this early developmental stage.

Materials and Methods

Broodstock Collection

Sexually mature *A. vulpes* (n = 13 females; n = 6 males) were collected by hook and line in a pre-spawning aggregation in November 2019 in Great Abaco, The Bahamas. Precise locations are not being published to ensure protection of the aggregation from poaching and other damaging activities. The reproductive state of females was assessed by cannulation and subsequent assessment of oocytes using a Dino-Lite digital Edge AM4815ZTL microscope. Females with oocytes >700 μm in diameter (Mejri et al., 2019a) were kept and utilized in the spawning trials. Males were assessed by palpating the abdomen and observing the presence/absence of milt. The males that presented free-flowing milt were also selected (Mejri et al., 2019a). These fish were transferred to temporary holding facilities aboard the research vessel M/Y *Albula* and held in four 1500 L circular tanks, which were shaded and supplied with aerated flow-through seawater under ambient light conditions.

Hormonal Induction and Spawning

In total, eight females (n = 8) were determined to be at an appropriate stage of reproductive maturity for attempted hormonal induction. Following protocols described by Mejri et al. 2019a, six females received initial intraperitoneal injections of 20 mg/kg of carp pituitary extract (CPE; Stoller Fisheries, Spirit Lake, IA, USA). The cannulation

was repeated after 24 h and a second injection of CPE (20 mg/kg) was administered, if, after 24 h, gentle pressure applied to their abdomens did not result in release of gametes. As controls, two females (n = 2) received only 1 mL injections of physiological saline (sham injections). To minimize handling stress during cannulation, injection, and egg extrusion, fish were held in a canvas tournament fish carry bag filled with seawater containing the anesthetic MS-222 (1 g/L) until they were lightly sedated. Eggs were collected in a dry bowl, sperm from 2 to 3 males was directly added to the eggs, and 500 mL of seawater was used to initiate fertilization. After 2 min, eggs were gently rinsed with sterile seawater using a flow through rinsing bucket to remove any sperm excess. Fertilized eggs were transferred to incubation tanks, and all broodstock were returned to their holding tanks to recover.

Incubation, Hatching, and Rearing

For incubation on the M/Y *Albula* research vessel, ambient seawater was pumped through a 1 μm filter sleeve into a reservoir where it was first sterilized using sodium hypochlorite and then neutralized by addition of sodium thiosulfate. Eggs and larvae were incubated in both 6 L aerated jars and in Kreisel tanks. Fertilized eggs from each female were incubated separately. The jars were placed in an insulated and heated water bath to maintain a temperature of ~25 °C. Kreisel tanks were circulated through a heated and aerated 20 L sump. Flow was adjusted to a level sufficient to circulate the eggs without clogging the drain screen.

Water exchanges were conducted with sterile pre-heated seawater as needed to remove dead eggs from the bottom of the incubation containers. Hatching occurred between 25 and 28 h after fertilization and larvae were incubated in the same Kreisel tanks. At approximately 23–25 h post hatch (hph), eggs were placed in oxygen permeable Kordon breathing bags and into insulated coolers for approximately 5 h for transport from the ship to the aquaculture facility at Harbor Branch Oceanographic Institute, where Kreisel tanks (n = 12) were submerged in a large recirculating aquaculture system. The larvae from both females were pooled and distributed randomly over the 12 tanks. The Kreisels were fed with small submersible magnetic drive pumps and drained back into the system. The system was outfitted with aeration, oxygen injection, a fluidized bed biofilter, and an inline heater. Water quality was measured twice daily, dissolved oxygen was kept above 100% saturation and temperature maintained between 26 ° and 28 °C. In anticipation of the exhaustion of endogenous nutrition, a series of potential diets were prepared to mimic the flocculent nature of marine snow: preparations of 19.0 g of macerated shrimp and squid, mixed with 0.5 g of a commercial feed mix (INVE Selco S.parkle), 0.5 g of pig gelatin, and 20 mL of water (this mixture was briefly heated and allowed to set, then lyophilized and stored frozen prior to rehydration into a mucilaginous material), as well as flake aquarium fish food soaked in water prior to introduction into the tanks. These diets ranged widely in nutritional quality, like marine snow.

Sampling and Microscopy

An initial egg sample (n ≥ 20) was collected at fertilization, and larvae (n ≥ 10) were sampled at 50% hatch and at every 4 to 7 h post-hatch (hph) for 192 h. Sampling ended when all larvae had died. A subsample was stored in 10% NB formalin for image analyses and another subsample was stored in cryovials and preserved at −80°C for lipid analyses. Total length (TL), yolk sac size (D_Y), and oil droplet diameter (D_o) were measured and photographed using an OLYMPUS BX51 microscope with cellSens digital imaging software at magnifications between 20× and 100×.

Lipid Analyses of Larvae

Lipids from a pool of eggs (n = 2, [100-200 mg] at fertilization) and larvae (n = 2 [20–100 mg]) wet weight from each time-point sample were extracted and analyzed according to procedures developed by Folch et al. (1957) and modified by Parrish (1999) (Folch et al., 1957; Parrish, 1999). Briefly, samples were homogenized in a dichloromethane: methanol: water (2:1:1) solution and centrifuged three times. The total amount of lipid extract was suspended in 100% dichloromethane for subsequent lipid class analysis. Lipid classes were determined using thin layer chromatography and flame ionization detection (Iatroscan MK 6s analyzer, Iatron Laboratories Inc., Tokyo, Japan). Samples were spotted in duplicate onto a series of silica gel S4 type Chromarods (5 μm particle size) and developed in a four-solvent system of different polarities to allow for lipid class separation (Parrish, 1987, 1999). The Iatroscan MK 6s was calibrated using known compound classes from standard in the range of 1–10 μL (a mixture of: n-Nonadecane, 99%; Cholesteryl stearate, 96%; 3-Hexadecanone, 95%; Cholesterol, 95% [Thermo Fisher Scientific, Sigma-Aldrich, MO, USA]; Glyceryl tripalmitate; Palmitic acid; L-a-Phosphatidylcholine [Sigma-Aldrich, USA]; 1-O-Hexadecyl-rac-glycerol [BACHEM America Inc., CA, USA]) and peaks were quantified using Peak 453 software.

The contribution of each lipid class was standardized to mg lipid g^{-1} wet weight (WW). The sum of the amount of all the lipid classes in each sample provided the total lipid content (mg/g WW), while each lipid class was measured as percent of total lipids. Lipid classes were grouped as 'storage' or 'structural', depending on their primary functional roles. Ketones (KET), triacylglycerols (TAG),

wax esters-steryl esters (WE-SE), free fatty acids (FFA), hydrocarbons (HC), and sterols (ST) were combined as the 'storage' lipids, and acetone-mobile polar lipids (AMPL), and phospholipids (PL) as the 'structural' lipids. Wax esters and steryl esters were considered together in this study as WE-SE, since the method used does not allow the separation of the two lipid classes.

Ethics Statement

This work has been carried out under the Bahamas Department of Marine Resources permit number MAMR/FIS/17 and ethical approval was received from Florida Atlantic University's Institutional Animal Care and use Committee (IACUC, protocols A16-34 and A19-36).

Statistical Analysis

A permutational ANOVA (PERMANOVA with 9999 permutations), including *a posteriori* pairwise comparison, was performed on lipid classes and total lipids. Specifically, a 1-factor PERMANOVA was performed to assess differences during early larval development. Assumptions of multivariate homoscedasticity were verified with a PERMDISP test, and data were transformed (arcsine square root of percentages) when necessary. SIMPER analyses were run to analyze the similarity in lipid class profiles, using a Bray–Curtis similarity matrix with PRIMER 7 (v. 7.1.12) and PERMANOVA+ (v.1.0.2). Lipid classes identified by SIMPER analysis that explain most of the differences between treatments were tested with one-way ANOVA. Assumptions of homoscedasticity and normality were tested with Levene's and Shapiro–Wilk tests, respectively. A trend analysis using stepwise regression was conducted with larval length, yolk sac size (D_Y), and oil globule diameter (D_o) as the dependent variables and time, time^2, and time^3 as the independent variables to determine the best-fit model for growth. In addition, linear regressions were used to determine larvae growth from 0 to 75 hph (Proc Reg, SAS 9.4, SAS Institute Inc., Cary, NC). A Pearson correlation analysis between all response variables has been performed.

Results

Leptocephalus Growth and Development

Although all hormone-injected females had oocytes that survived through the final maturation process, only three females completed the oocyte maturation, ovulating hydrated oocytes from ovarian follicles into the abdominal cavity and were successfully strip-spawned. Neither of the **control fish underwent final oocyte maturation or ovulation. Eggs from two females successfully** developed and hatched. At hatch, the mean length of the bonefish leptocephalus larvae was 4162 ± 228.8 µm (mean ± SD) (Fig. 1). The average yolk sac size was 822 ± 126.7 µm and average oil droplet diameter was 254 ± 32.7 µm (Fig. 2).

Larvae hatched with no eye pigmentation, a closed mouth, and no apparent digestive system. Hourly growth trajectories from hatch to 75 hph were initially rapid and linear, as demonstrated by the linear regression equation (n = 44) (Fig. 1, inset). However, the overall larval growth from hatch to 192 hph showed a nonlinear trend (Fig. 1, main), as demonstrated by the nonlinear regression equation (n = 73) for the overall post-hatch period. The larvae total length increased by approximately 54.9 µm/h (1.32 mm/day). From this point, growth slowed down to 14.2 µm/h (0.34 mm/day) until 192 hph, when all larvae perished. Larvae had intact oil droplets until 75 hph (Fig. 3a) and were observed to consume particles of all diets beginning at 120 hph (Fig. 3b), but the diets proved to be insufficient for continual growth and survival (Fig. 3c). Yolk sac size decreased by 3.72 µm/h (0.09 mm. day^{-1}) from hatching to 192 hph (Fig. 2a). The oil droplet size did not change during the first 75 hph, but it decreased rapidly by 1.69 µm/h (0.04 mm/day) from 75 hph onward (Fig. 2b). Yolk sac size followed a quadratic regression model; however, the oil droplet diameter followed a cubic regression model.

Total Lipid Concentration and Lipid Class Composition

Total lipids (TL) and relative percentages of lipid classes varied significantly during early larval development from 0 h post fertilization (hpf) to 192 hph (Pseudo-$F_{(11,20)} = 11.48$, $p = 0.001$). The trend in lipid classes following important stages in leptocephalus larvae development was interesting. As shown in Fig. 4, the n-MDS analysis showed a separation of the data into four groups, which represent stages of development. The first stage was egg samples at 0 hpf that were characterized by higher levels of WE-SE and total lipid content (33% of total lipids [TLS] and 23 mg/g WW, respectively; Table 1). The second stage consisted of samples from 4 to 45 hph coinciding with the hours post hatch when the oil droplet is still almost intact. Here, both total lipids and percentages of WE-SE decreased to an average of 12 mg/g WW and 4% of TL, respectively (Fig. 4 and Table 1). This stage is also marked by an increase of HC and AMPL relative percentages. The third stage consists of larvae samples from 53 to 120 hph, which marks the period where the oil droplet started decreasing in size. Although the same pattern as in stage

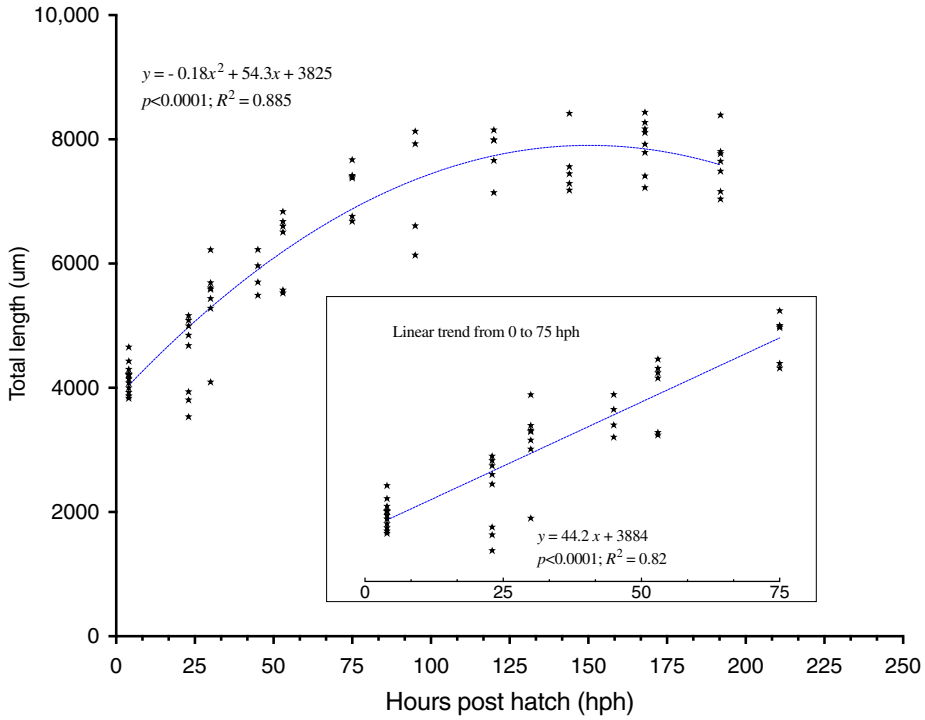

Fig 1 Trend analysis using stepwise regression showing a nonlinear bonefish (*Albula vulpes*) larval length in relation to hours post hatch (hph). Extracted data, from 0 to 75 hph (inset) shows linear growth during the first 75 hph. Data are presented as mean ± SD, and n ≥ 10

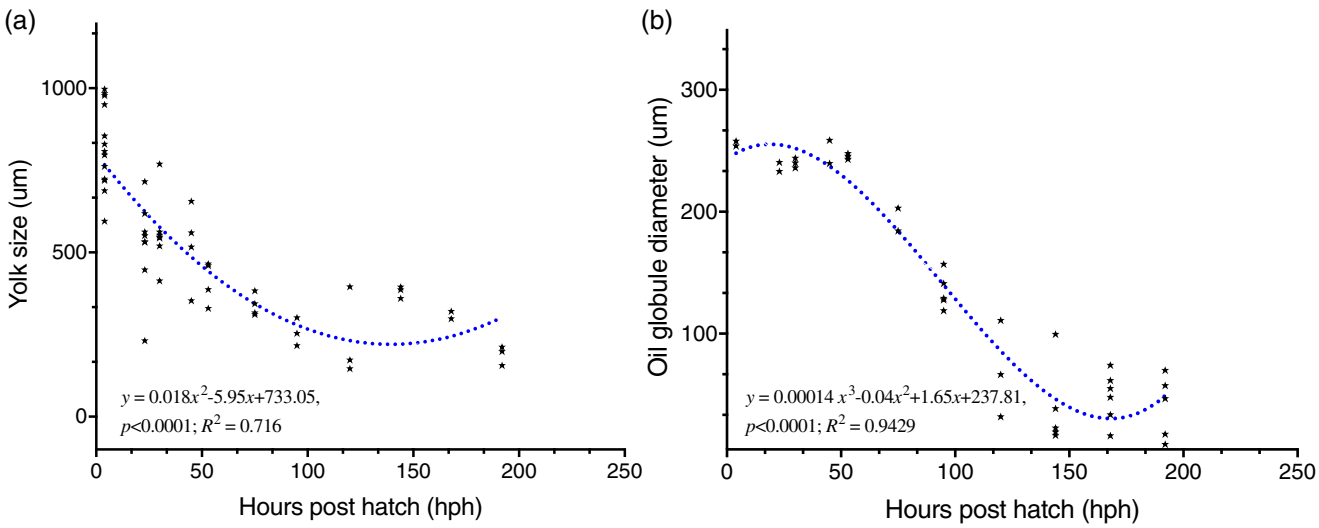

Fig 2 Trend analysis using stepwise regression showing a non-linear regression models with 95% confidence interval with hours post hatch (hph) in relation to (a) yolk size (quadratic regression model), and (b) oil droplet diameter (cubic regression model) of bonefish (*Albula vulpes)* leptocephalus larvae. Data are presented as mean ± SD, and n ≥ 10

two is observed regarding WE-SE, HC, and AMPL, this stage is marked by the important decrease in ST relative percentages by almost five times from the previous stage (*i.e*. 4 to 45 hph). The last stage consists of samples from 144 to 192 hph (samples corresponding to 2nd, 3rd, and 4th days of exogenous feeding) that show complete depletion of WE-SE, a slight decrease in PL, and an increase in FFA, and KET relative percentages. A significant correlation between WE-SE, ST, PL, yolk length, oil diameter, larvae length, and hours post hatch was observed (Table 2). All variables were negatively correlated with hours post hatch and larvae length. The three lipid classes (WE-SE, ST, and PL) were on the other hand, positively correlated with oil diameter and yolk length. For more details about

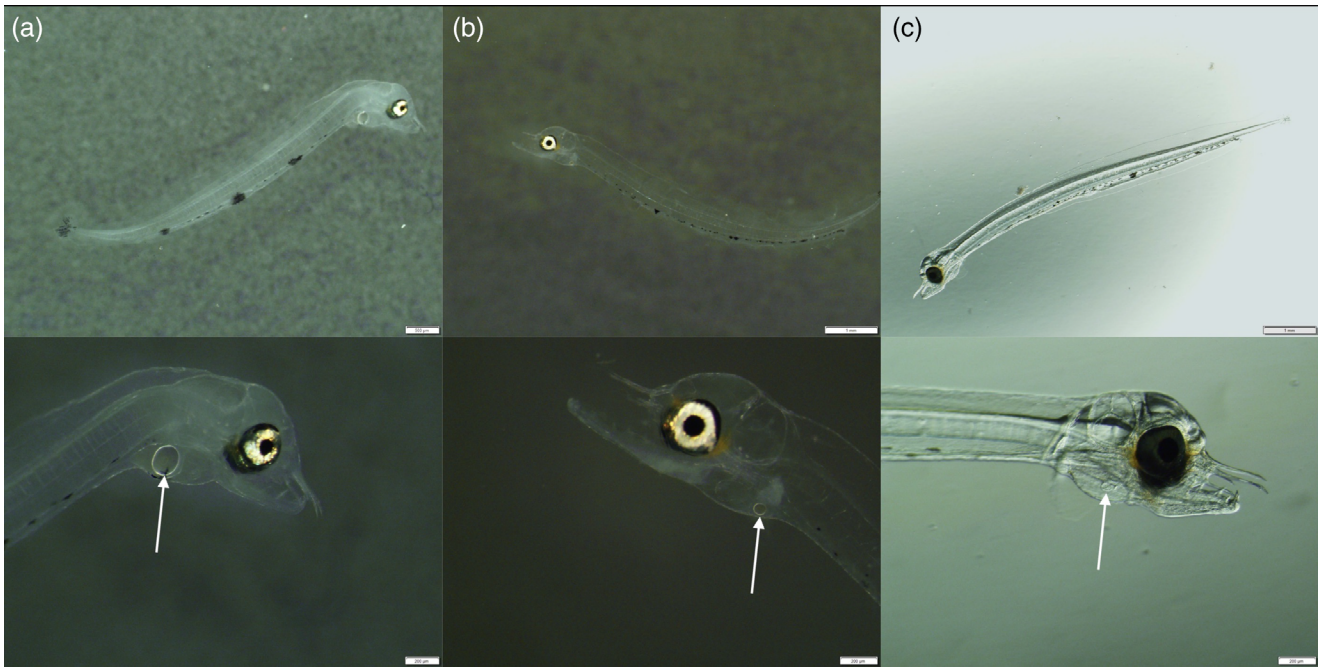

Fig 3 Photomicrographs taken at varying stages of bonefish (*Albula vulpes*) leptocephalus larval development. *Top*: Full photographs of larvae; bottom: Close-up of head region. From *left to right*: (a) 75 hph (hours post hatch), (b)120 hph (start of exogenous feeding), and (c) 192 hph. Photos show a steady decrease in oil droplet (at arrow) size after 75 hph

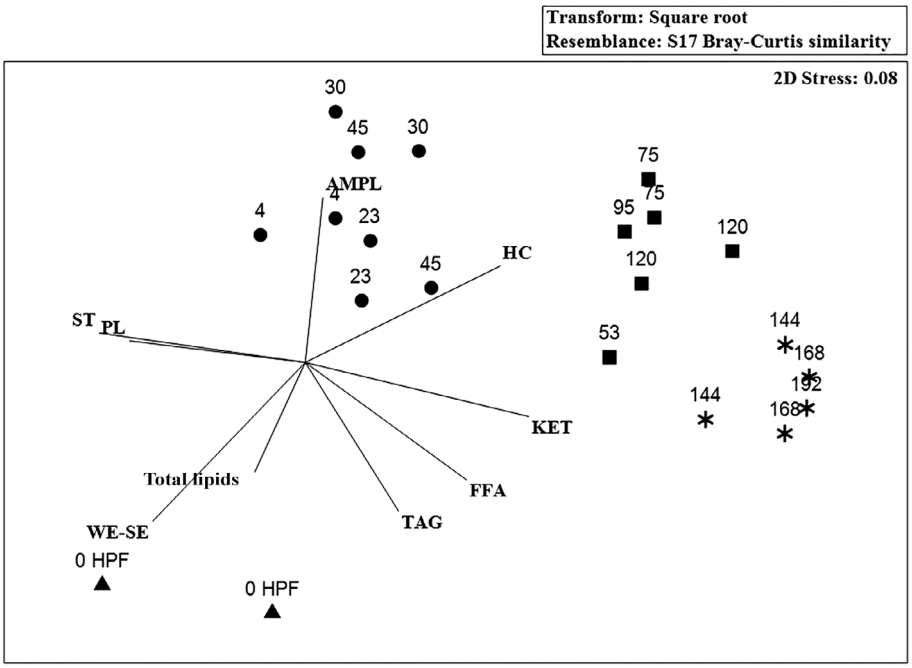

Fig 4 Non-metric multidimensional scaling of the bray–Curtis similarity matrix based on the relative abundance of lipid classes profiles and total lipids associated with eggs at 0 h post fertilization (hpf) and larvae sampled during the 192 h post hatch (hph). The n-MDS shows four clusters: 1st cluster (triangles) regroups egg samples at 0 hpf; the 2nd cluster (circles) regroups samples from 4 to 45 hph coinciding with the hours' post hatch when the oil droplet is still almost intact; the 3rd cluster (squares) comprises larvae samples from 53 to 120 hph, which marks the stages where the oil droplet starts decreasing in size. The 4th cluster (stars) regroups samples from 144 to 192 hph (samples corresponding to 2nd, 3rd, and 4th days of exogenous feeding). The arrows represent the lipid class responsible for most of the variation. AMPL, acetone-mobile polar lipids; FFA, free fatty acids; HC, hydrocarbons, KET, ketones; PL, phospholipids; ST, sterols; TAG, triacylglycerols; WE-SE, wax esters-steryl esters

Table 1 Total lipid and lipid class relative percentages (mean ± SD, n = 2) of bonefish (*Albula vulpes*) leptocephalus eggs at 0-h post fertilization (hpf), and larvae at 4, 23, 30, 45, 53, 75, 95, 120, 144, 168, and 192 h post hatch (hph)

Lipid classes	Hours post hatch											
	0 hpf	4	23	30	45	53	75	95	120 (day 1*)	144 (day 2*)	168 (day 3*)	192 (day 4*)
Neutral fraction												
HC	**6.63 ± 2.22b**	**15.67 ± 4.84ab**	**16.96 ± 2.47ab**	**22.40 ± 3.05ab**	**15.49 ± 0.05ab**	**24.68 ± 0.00ab**	**33.40 ± 0.68a**	**27.69 ± 0.00ab**	**29.98 ± 10.33a**	**23.39 ± 9.18ab**	**26.73 ± 5.69ab**	**28.48 ± 0.00ab**
WE-SE	33.72 ± 2.50a	5.93 ± 1.13b	4.78 ± 0.21bc	1.73 ± 0.18cd	2.59 ± 0.26bcd	3.14 ± 0.00bcd	1.94 ± 0.61bcd	2.98 ± 0.00bcd	2.92 ± 0.40bcd	0 ± 0.00d	0 ± 0.00d	0 ± 0.00d
KET	3.97 ± 1.15cde	2.03 ± 0.32e	3.50 ± 2.02de	3.00 ± 0.16de	7.95 ± 1.87bcde	11.38 ± 0.00abcd	10.41 ± 1.60abc	12.84 ± 0.00ab	12.66 ± 3.14ab	13.55 ± 1.29ab	16.80 ± 0.67a	17.91 ± 0.00a
TAG	6.38 ± 1.14	4.12 ± 0.62	4.44 ± 0.18	4.04 ± 0.94	4.35 ± 0.18	3.03 ± 0.00	4.99 ± 0.16	4.51 ± 0.00	5.15 ± 2.43	7.78 ± 0.44	6.52 ± 0.23	6.73 ± 0.00
FFA	1.83 ± 0.38e	1.18 ± 0.75e	3.92 ± 0.46bcde	0.42 ± 0.59e	1.50 ± 2.12e	8.23 ± 0.00abc	1.32 ± 0.66e	1.59 ± 0.00de	2.99 ± 0.46cde	6.22 ± 0.27abcd	9.13 ± 1.63a	8.89 ± 0.00ab
ST	**14.48 ± 9.19abcd**	**19.41 ± 0.38a**	**16.27 ± 2.53abc**	**15.37 ± 0.04abcd**	**17.23 ± 1.55ab**	**3.48 ± 0.00abcd**	**2.05 ± 0.97d**	**1.73 ± 0.00bcd**	**2.83 ± 0.13cd**	**3.16 ± 0.03cd**	**4.66 ± 0.64bcd**	**5.52 ± 0.00abcd**
Polar fraction												
AMPL	**19.90 ± 4.95**	**42.34 ± 4.35**	**36.74 ± 5.53**	**41.26 ± 2.57**	**34.76 ± 4.83**	**30.27 ± 0.00**	**37.42 ± 1.24**	**39.87 ± 0.00**	**38.37 ± 11.68**	**39.30 ± 8.96**	**30.86 ± 5.77**	**27.34 ± 0.00**
PL	**13.07 ± 3.17abc**	**9.29 ± 0.19abcd**	**13.36 ± 2.59abc**	**11.75 ± 1.73abcd**	**16.10 ± 1.10a**	**15.76 ± 0.00ab**	**8.43 ± 1.51bcd**	**8.76 ± 0.00cd**	**5.07 ± 0.08d**	**6.57 ± 0.82cd**	**5.26 ± 0.62d**	**5.09 ± 0.00cd**
Total lipids (mg/g WW)	23.71 ± 5.62a	13.23 ± 2.9b	12.56 ± 1.0ab	10.09 ± 0.88ab	14.72 ± 1.18ab	19.97 ± 0.00ab	19.93 ± 0.34ab	16.76 ± 0.00ab	14.27 ± 0.22ab	14.23 ± 1.47ab	13.38 ± 5.23ab	12.50 ± 0.00ab

Eggs hatched at 26 hpf, which means at 4 hph, there is 30 hpf. *means after start of exogenous feeding.

Numbers in bold face are the predominant lipid classes in bonefish leptocephalus larvae during early larvae development. Letters indicate values significantly different from one another (single factor ANOVA by means). A response variable without superscripts indicates that all means in that particular row are not significantly different from one another.

Lipid class abbreviations: AMPL, acetone-mobile polar lipids; FFA, free fatty acids; HC, hydrocarbons; KET, ketones; PL, phospholipids; ST, sterols; TAG, triacylglycerols; WE-SE, wax esters-steryl esters.

Table 2 Pearson correlation coefficients (n = 21) between response variables

	Larvae length	Oil diameter	Yolk length	WE_SE	ST	PLS
Hours post hatch	0.93	−0.96	−0.95	−0.53	−0.74	−0.74
Larvae length	1	−0.87	−0.99	−0.64	−0.83	−0.65
Oil diameter	−0.87	1	0.89	0.38	0.72	0.82
Yolk length	−0.99	0.89	1	0.63	0.82	0.67

We have reported here only the most significant correlations. All coefficients listed here have a $p \leq 0.001$.

Lipid class abbreviations: PL, phospholipids; ST, sterols; WE-SE, wax esters-steryl esters.

all variables correlation, please refer to the supplementary file (Table S1).

Discussion

The data denote a pattern of lipid metabolism by bonefish leptocephalus larvae with a specific use and conservation of certain lipid classes. Overall, a decrease in total lipids content and WE-SE proportions was shown over ontogeny. A decline in total lipids by 44% took place from fertilization to 4 hph and was marked by an 80% decrease in WE-SE proportions. This net decline in total lipids and WE-SE suggests that WE-SE was the main energy source during embryogenesis, whereas TAG levels did not change and were most likely conserved for later use. Older larvae of 75 to 192 hph were characterized by extremely low proportions of WE-SE, with numerous individuals with no reserves (*i.e*. larvae at 144, 168, and 192 hph). A summary figure of the important developmental milestones, illustrating the pattern of growth associated with lipids use has been added (Fig. 5).

After hatching, the decrease in total lipid content, in addition to the changes observed in lipid class content (mainly WE-SE) reflected the utilization and mobilization of lipids as substrates for energy and as precursors for biomembranes in the body of the larvae at an early stage (31 h post fertilization; from 0 to 4 hph). Such a strategy is indicative of a selective bulk intake of lipids from the yolk. The reason for such a selective lipid class recruitment is at present unknown, leaving an intriguing question for further study. A possible hypothesis could be that WE-SE are longer-term energy reserves and more buoyant than other neutral lipid classes such as TAG (Deibel et al., 2012). A recent study conducted by our group (Lombardo et al., n. d.) found that bonefish descend to 137 m as part of spawning behavior, and spawn at the pycnocline at 75 m. Such reproductive behavior might explain why bonefish eggs are rich in WE-SE; this may help the eggs stay buoyant until they hatch and develop into more effective swimmers.

Other studies about lipid mobilization by the early larvae of other species with oil globules, high lipid contents, tropical/temperate water habitats, and short periods of development such as red drum (*Sciaenops ocellatus*) (Vetter et al., 1983), Florida pompano (*Trachinotus carolinus*) (unpublished data), seabream (*Diplodus sargus*) (Cejas et al., 2004), and gilthead sea bream (*Sparus aurata*) (Mourente and Odriozola, 1990; Rønnestad et al., 1994) showed a sequential utilization of TAG, WE-SE, and PL from hatching until larvae started exogenous feeding. In this study, during the larvae stage from 4 to 192 hph, there seemed to be only small quantitative changes in the lipid content of the whole larvae. This supports the notion that amino acids could be catabolized for energy metabolism during this stage, as suggested by Finn (1994) for fish eggs containing oil globule(s) (Finn, 1994). This is also supported by our previous findings of rich compositions of free amino acids in bonefish oocytes collected from several islands in the Bahamas (Mejri et al., 2019b). Lipid classes profiles, following hatching, showed an interesting trend by segregating the data into three clusters. The total lipid concentration, ranging from 1% to 1.9% of WW was two times higher than type II leptocephali (larvae that have undergone metamorphosis) collected in the Gulf of Mexico, ranging from 0.3–0.7% of WW, with lengths between 20 and 200 mm (Deibel et al., 2012). This supports the hypothesis that type-I leptocephalus larvae (pre-metamorphic) accumulate more lipids than type-II larvae, in preparation for the energy demands of metamorphosis, and that large deposits of GAG are characteristics of older leptocephali, as suggested by the Deibel et al. (2012) study (Deibel et al., 2012).

Initial rapid larval growth rates (from 4 to 75 hph) in this study were similar to those previously observed in bonefish leptocephali (Halstead et al., 2020) and leptocephali of eels (Miller, 2009). The increase in HC proportions during this period, which are produced by the hydrolysis of simple and complex lipids, could be due to an increase in energy demand by the larvae. Given that the oil droplet size did not change during this period, we suspect that the hydrolysis of HC comes from lipids in the yolk. Larvae growth rates declined after 75 hph, coinciding with a decrease in

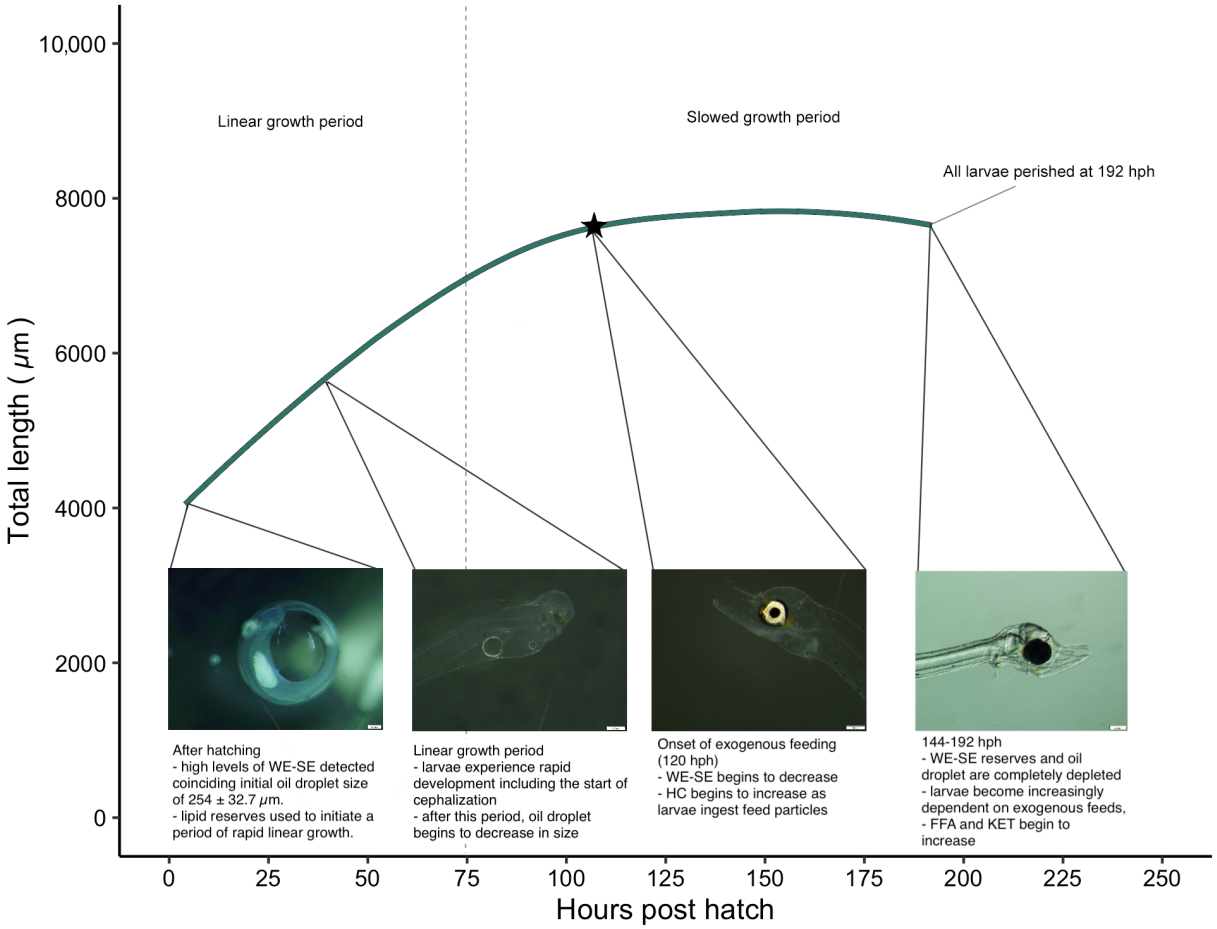

Fig 5 Summary figure showing developmental milestones on the overall plot trend of total length data over time. Photos are included to illustrate physical changes in larvae (captions inset). The star symbol shows the onset of exogenous feeding and the dashed line represents the end of the linear growth period and beginning of the slowed growth period. FFA, free fatty acids; HC, hydrocarbons; KET, ketones; WE-SE, wax esters-steryl esters

oil globule size. This period of development was marked by a switch in the energy allocation strategy, with ST concentrations being depleted until the end of the development period observed in this study. Such an observation suggests that ST serve as the major lipids that provide the basic matrix of the cellular membranes (Spector and Yorek, 1985; Stubbs and Smith, 1990; Vance and Vance, 2008). One of the limitations of this study is that we did not collect data for egg quality, which might suggest that the early larval performance and thus kinetics of lipids utilization could be affected due to the latter (Rainuzzo et al., 1997). However, based on field observations, after fertilization, we noticed that 80% of the eggs were buoyant and healthy. There was little evidence of unfertilized eggs in field notes and images taken at the time of collection. As larvae were incubated in Kreisel tanks for the duration of the experiment, underdeveloped or unhatched eggs were rarely found in the system. Some larvae did not hatch with deformities but later developed them over time (such as body kinks). In similar studies regarding the eel leptocephalus conducted in Japan, these deformities are common during rearing and are thought to be due to the current within the tank. Due to unique constraints during field collection at sea, data about egg survival, hatching rates, and fertilization rates were not recorded but will be taken into consideration in future trials.

During the exogenous feeding period, which lasted three days, almost all the larvae stopped at a chunk of a flake and/or the macerated shrimp/squid and repeatedly touched the particle. After several encounters with the food particles, the larvae opened its mouth and pressed on the particle before attempting to swallow it (*observations by Cianciotto during feeding*). Based on a frame by frame review of videos of the feeding leptocephalus it appeared that small fragments of the somewhat large particles that broke off were moving into the gut, but the larvae did not appear to be able to ingest whole particles. The reason that active feeding has been discounted in leptocephali seems to be the assumption that the gut of the leptocephali is not functioning and has minimal ability to absorb food (Hulet

and Marshall, 1978; Moser, 1981; Padrón et al., 1996; Pfeiler, 1986, 2000). This may not be true, at least for bonefish leptocephali. Judging from the observed presence of certain particles in the gut and the relative increase of free fatty acids proportions, some small molecules of food and water must have been absorbed. In addition, giving the variance nutritional quality of the exogenous diets, some larvae might have assimilated more nutrients than the others. In a future study, we aim to conduct feeding trials with less diets and more replications to determine how nutritional quality of diets is affecting growth and survival of the leptocephalus.

In conclusion, our interest in the lipid and lipid class content of bonefish leptocephali (type-I larvae) was primarily to understand their nutrition, energy storage and mobilization, growth rate, and thus their ecological and physiological adaptations at the beginning of an extended larval period. We determined relatively high total lipid concentration prior to hatching, of which 40% was used during embryogenesis. The main component of lipid metabolism seemed to occur during the first part of yolk-sac stage, with WE-SE being selectively catabolized. Growth rate slowed beyond 75 hph and, judging from the constant total lipid content, free amino acids might be catabolized as well during this period. In addition, the decrease of the relative proportions of neutral lipid ST indicates that bonefish leptocephali were allocating energy toward somatic growth. Concurrently, judging by the absence of change in TAG relative percentages, they were preserving some energy stores to possibly increase their starvation tolerance at the beginning of exogenous feeding. Finally, although the exogenous feeding trials were not successful, whatever their natural diet might be, certain kinds of paste food with an emulsion characteristic should be a promising diet (Chow et al., 2019; Mochioka et al., 1993; Tanaka et al., 2003). The latter were prepared in our lab and will be used in future feeding trials over the next spawning season.

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Author contributions S.M. and A.A. were responsible for the oversight of the project, development of main ideas, and experimental design. S.M. oversaw laboratory analysis and aided with field collection, spawning induction, data analysis and manuscript preparation. P.S.W., J.S., and A.J.A. were responsible for securing project funding as well as directing field work and manuscript preparation. J.M.S. was responsible for securing project funding, field collection, manuscript preparation, broodstock management, and designing larval rearing systems. A.C.C. and C.R. were responsible for the experimental design, maintenance of the larval rearing system, and manuscript editing. V.U. conducted laboratory analysis on samples and aided with manuscript preparation. P.S.W. was responsible for data analysis, broodstock management, experimental design, and manuscript preparation.

Data Availability Statement

Data available by request. Contact corresponding author.

Conflict of Interest All authors declare that they have no conflicts of interest.

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REVIEW
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Essential Fatty Acid Requirements in Tropical and Cold-Water Marine Fish Larvae and Juveniles

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To improve survival at early developmental stages (larvae and juveniles) of captive fish species, essential nutrients [i.e., essential fatty acids (EFA)] need to be identified. The physiological needs are likely to be different among species, particularly among those using different thermal habitats, because lipids are largely used to maintain cell membrane integrity (homeoviscous adaptation) in fishes. This review paper will focus on currently published research and the main results from our laboratories regarding optimum qualitative EFA requirements during larval and early juvenile stages in a warm-water marine species, the Florida pompano (*Trachinotus carolinus*), and a cold-water marine species, the winter flounder (*Pseudopleuronectes americanus*). To identify the qualitative optimal EFA requirements, we calculated the ratio of certain fatty acids (FA) in larval or early juvenile tissues to total FA present in the diet. This ratio indicates whether a specific FA from prey is selectively incorporated by larvae and juveniles. Overall, we found that young larvae from both cold- and warm-water species have greater demands for n-3 and n-6 highly unsaturated fatty acids (HUFA) than do larvae at weaning stages. However, the qualitative EFA requirements of the cold-water species at all early developmental stages were higher than those of the warm-water species. Enriched rotifer diets provided satisfactory amounts of omega 3 and omega 6 in Florida pompano, with small selective retention for docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA), suggesting a potential minor diet deficiency in these EFA. There were higher deficiencies in the cold-water species fed enriched rotifers, as demonstrated by the higher selective retentions of all EFA (DHA, EPA, and ARA), with the exception of larvae fed with copepods. The physiological needs in EFA for juvenile development seemed to be better met for both species when they were fed micro pellets. From the beginning of settlement and in young juveniles, qualitative values of 12% DHA, 10% EPA, 5% ARA, and 40% PUFA of total FA seem to be required for winter flounder juvenile development. In Florida pompano, these requirements could be met until larger juvenile stages, with 15% DHA, 3% EPA, 2% ARA, 2% DPA, and total PUFA below 30% of total FA. This review was done to aid future research aiming to develop nutritionally balanced microdiets or live-prey enrichment diets to satisfy the physiological requirements of captive tropical and cold-water marine fish species.

Keywords: warm-water fish, cold-water fish, essential fatty acids, larvae, juveniles, diet

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INTRODUCTION

Lipid energy is transferred from phytoplankton to fish *via* zooplankton. A good indication of the energetic importance of lipids for fish was demonstrated by Yaragina et al. (2000), who showed that total lipid energy is a predictor of reproductive potential in fish stocks. During digestion, ingested lipids containing fatty acid chains of more than 14 carbons are hydrolyzed to yield free fatty acids, monoacylglycerols, and – in the case of phospholipids (PL) – lysophospholipids. These products pass through the mucosal wall of the small intestine, reform into triglycerides, and are transported to tissues by chylomicrons in the blood (Budge et al., 2006). Small-chain fatty acids (<14°C) are transported directly to the liver to be oxidized. Thus, long-chain fatty acids can be preserved during the digestive process and selectively retained by fish to sustain later development.

Aquaculture development of new marine fish species is challenged by a critical knowledge gap concerning the nutritional needs at larval and early juvenile stages. The optimization of feeding conditions and the nutritional quality of starter diets, which are generally at least partially made up of enriched live prey, directly influence the success of these early stages as estimated by growth and survival surveys. Lipids are a major nutritional constituent and are the main source of metabolic energy sustaining the rapid development of early stages (Glencross, 2009). Lipids provide at least two-thirds more energy per gram than proteins or carbohydrates (Parrish, 2013). Of the different lipid classes, neutral lipids, including wax esters, sterols, free fatty acids, and triglycerides have a rapid turnover and can satisfy short-term energy needs (Budge et al., 2006), while the β-oxidation of saturated fatty acids (SFA) releases energy more efficiently than polyunsaturated fatty acids (PUFA; Langdon and Waldock, 1981).

Despite years of research, lipids remain the least well-understood nutrient (Glencross, 2009; Parrish, 2013). Some fatty acids are considered as essential fatty acids (EFA) for marine fish development because they cannot be biosynthesized to support normal development (Glencross, 2009). EFA are involved in different physiological functions, including reproduction, immunity, ion balance regulation, muscle contraction, cell adhesion, vascular tone, buoyancy control, and brain and eye development, and thus directly affect the growth and survival of marine animals (Glencross, 2009; Pond and Tarling, 2011; Gurr et al., 2016). These EFA are mainly from two related families (n-3 and n-6) and from three long-chain PUFA: docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (ARA, 20:4n-6), which have long been considered crucial. DHA and EPA have important roles in the maintenance of membrane fluidity while ARA and EPA are precursors of bioactive eicosanoids (Tocher, 2010). During larval development, DHA is preferentially incorporated into nervous and retina tissue (Izquierdo et al., 2000; Villalta et al., 2008), and growth anomalies and high mortality are observed when DHA is insufficient (Tocher, 2010). Other dietary FA, including linoleic acid (LA; 18:2n-6) and α-linolenic acid (LNA; 18:3n-3), are stored in muscle tissue to meet physiological needs and are also related to fish growth and survival to some extent (Jardine et al., 2020). These FA are precursors of DHA, EPA, and ARA, but as already pointed out; the activities of the specific enzymes (desaturase and elongase) responsible for their biosynthesis are limited in most marine species.

Artemia and rotifers contain high levels of PL, which are considered valuable for fish nutrition (Tocher et al., 2008), and they are the main live prey used in aquaculture. However, their low levels of EFA are not adequate for early life stages, so they must be enriched before being used as prey (Øie et al., 2011). In contrast, copepods, which are natural fish prey in the wild, are rich in phospholipids and EFA, but their laboratory production is complex and challenging (Drillet and Lombard, 2015). Although the PL content per dry weight (DW) in rotifers and *Artemia* cannot be manipulated, their fatty acid composition can be modified with the use of enrichments (Castell et al., 2003; Monroig et al., 2003; Seychelles et al., 2009; Hawkyard et al., 2015).

One interesting method used to determine the prey's nutritional quality in terms of EFA is the ratio of specific fatty acids in larval or early juvenile tissues to the total fatty acids present in the prey [the fish to diet (FD) ratio]. This FD ratio indicates whether a specific prey fatty acid is selectively incorporated by larvae and juveniles (Copeman et al., 2002; Pernet and Tremblay, 2004; Gendron et al., 2013; Martinez-Silva et al., 2018). If the relative proportion of a specific fatty acid in larvae or juveniles to their diet is equal to or below 1, then the specific requirement for this fatty acid could be considered as satisfied. In contrast, if the relative proportion is higher than 1, then we would presume that this FA is selectively incorporated by larvae or juveniles, which may indicate a potential dietary deficiency.

The objective of this review paper is to present published research regarding live-prey diets used in early developmental stages of Florida pompano (*Trachinotus carolinus*) and winter flounder (*Pseudopleuronectes americanus*), two contrasting models from tropical and temperate coastal habitats, respectively, to determine whether their ontogenic EFA needs are comparable. Both species are commercially important and contribute to the economies of their distribution range, compelling the aquaculture industry to search for more effective rearing methods. Our aim is to examine whether FD ratios can give information about differing needs through the first stages of development and how these needs are comparable between tropical and cold-temperate species. To estimate potential dietary deficiencies in EFA, we used the FD ratio as an optimization tool. The FD ratio as well as compositions of fatty acid that are considered to be essential or of special importance for larval and juvenile development [18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, 22:6n-3, total n-3, total n-6, SFA, monounsaturated fatty acids (MUFA), and PUFA] are presented and compared between relatively similar ontogenic stages in the two species considered (**Table 1**).

The lipid composition of larvae and/or juveniles has been presented in detail for Florida pompano (Cavalin and Weirich, 2009; Hauville et al., 2014, 2016; Rombenso et al., 2016, 2017;

TABLE 1 | Age or mass of larvae and juveniles of the two species compared in this review.

Ontogenic stage	Florida pompano	Winter flounder
Mouth opening	No data available	4 DPH
Live prey feeding	9 DPH	15–22 DPH
Weaning	17 DPH	26 DPH
Beginning of settlement		38 DPH
Early settled juveniles		30 DPS
0+ juveniles		60 and 90 DPS
Juveniles	Around 40 g	

Florida pompano (T. carolinus): larval rearing at 26–27°C; winter flounder (P. americanus): larval rearing at 10°C. DPH, days post-hatch; DPS, days post-settlement.

Jackson et al., 2020) and for winter flounder (Mercier et al., 2004; Seychelles et al., 2011, 2013; Vagner et al., 2013; Bélanger et al., 2018; Martinez-Silva et al., 2018). While these studies examined different developmental stages, we have merged the results for the purpose of comparison.

NUTRITIONAL RATIOS AND FATTY ACID COMPOSITION

FD Ratio in Larvae

Florida pompano larvae at the live-prey feeding period-fed several enriched rotifer diets [*Isochrysis* sp. (ISO), *Pavlova* sp. (PAV), Protein Selco Plus (PS+), Ori-Green (OG), Protein highly unsaturated fatty acid (HUFA; PH), and AlgaMac 3050 (AM)] showed specific needs for DHA, EPA, and ARA, with selective retention of these three EFA (**Figure 1**). The FD ratio for EPA was relatively close to one in larvae fed the different enriched diets, particularly for the AM- and OG-enriched diets, showing accumulations of up to 7% of total FA (**Table 2**). Exceptions were noted for ISO-enriched diets, which showed ratios near 1.5. DHA requirements seemed to be satisfied by most diets, with an average of 15% of total FA (**Table 2**) and the best FD ratio for DHA being observed in larvae fed diets enriched with PAV and PH (ratio = 1.053 and 1.056, respectively). This suggests that these diets satisfied most physiological needs of the larvae at this developmental stage. All diets showed FD ratios above one for ARA, suggesting that these enrichments did not satisfy the requirements for this PUFA (**Figure 1**). The maximal ARA accumulation observed at the live-prey feeding stage was 3.5% of total FA (**Table 2**), with FD ratios between 1.26 and 1.89 depending on the diet. LA and LNA were not selectively retained by larvae at this stage, with ratios < 1 and low relative percentages of LNA (0.6% of total FA; **Table 2**). However, two diets (PAV and AM) seemed to better supply the LA and LNA requirements, with FD ratios equal to one (**Figure 1**). Overall, total omega 3 (n-3) and omega 6 (n-6) FD ratios were close to one for all diets (**Figure 1**), suggesting that the different enriched rotifer diets provided satisfactory amounts of n-3 and n-6 PUFA for larvae at the live-feeding stage.

At the weaning period [17 days post hatch (DPH)], Florida pompano larvae were fed different microdiets (Gemma, Otohime, and LR803) and generally showed FD ratios close to one.

TABLE 2 | Relative percentages of fatty acids in Florida pompano (*T. carolinus*) larvae at live prey feeding and weaning (9 and 17 DPH, respectively; Cavalin and Weirich, 2009; Hauville et al., 2014).

	Live prey feeding	Weaning
18: 2 n-6 (LA)	6.58 ± 2.53	16.32 ± 10.45
18: 3 n-3 (LNA)	0.59 ± 0.28	2.22 ± 0.99
20: 4 n-6 (ARA)	3.55 ± 0.41	0.47 ± 0.15
20: 5 n-3 (EPA)	7.28 ± 1.22	5.20 ± 2.65
22:6 n-3 (DHA)	15.06 ± 4.59	8.57 ± 2.29
Total n-3	28.64 ± 2.31	–
Total n-6	14.80 ± 1.70	–
SFA	–	29.99 ± 1.87
MUFA	–	23.80 ± 3.87
PUFA	–	39.29 ± 6.37

Data represent mean ± SD from all diet treatments at 9 and 17 DPH. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

One exception was noted: EPA ratios were between 1.47 and 1.86, indicating selective retention for this EFA and potential diet deficiencies. Among the three microdiets, Otohime micro pellets followed by LR803 seemed to be the most appropriate; they fulfilled the physiological fatty acid requirements of larvae at the weaning stage, with a PUFA ratio equal to 1.01 for both diets (**Figure 1**).

In winter flounder larvae, overall larval contents in neutral EPA and DHA and in polar DHA were higher at the mouth-opening stage, reflecting the maternal nutritional contribution (yolk sac; **Table 3**). Overall, EPA and DHA contents were usually higher in polar than in neutral lipids, as reflected by the total n-3 value (**Table 3**). Once larvae started feeding, FD ratios for the neutral lipids showed that the three EFA (ARA, DHA, and EPA) were immediately retained (FD values much greater than one), regardless of the diet [rotifers enriched with Selco or days' post settlement (DPS)]. For most diets, the high retention levels of EFA in the neutral fraction were maintained throughout development, but larvae fed rotifers enriched with Sparkle or copepods showed ratios near or below one (**Figure 2**). This may be related to crucial biological needs, since EFA are important for physiological functions. Indeed, FD ratios for polar lipid fractions were mostly greater than one whatever the diet or the larval stage of development, except for larvae fed copepods at the live-prey feeding stage (**Figure 2**). For each EFA, the use of copepods as live prey decreased the FD ratio of polar lipids in larvae relative to diet content, but the ratio was slightly greater than one for ARA and EPA. In these larvae, overall LNA contents were usually very low. This is not surprising since *Artemia* were not included in the larval diets (**Table 3**), and *Artemia* are usually characterized by high LNA levels (Rocha et al., 2017). The requirements for omega n-6 seemed to be met regardless of the diet, as indicated by their elimination in neutral lipids and their incorporation into polar lipids at percentages lower than those found in the different diets (**Figure 2**). However, the sum of omega n-3 showed ratios suggesting potential feed deficiency (>1), particularly in the polar fraction, indicating that larval needs were not met except when copepods were used as live prey.

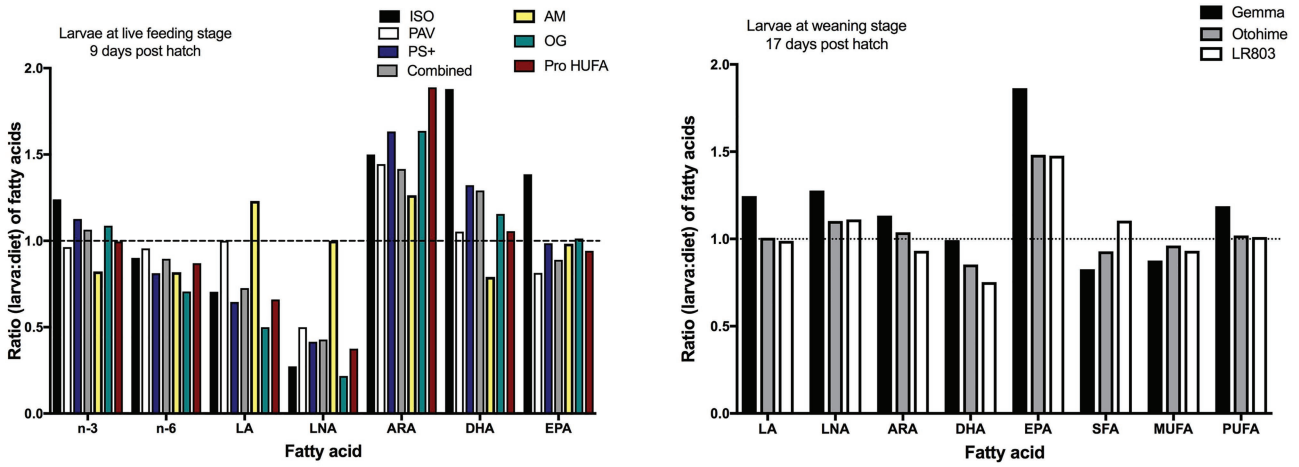

FIGURE 1 | Ratio (larva: diet) of fatty acids in Florida pompano (*Trachinotus carolinus*) larvae at the live feeding [9 days post hatch (DPH)] and the weaning period (17 DPH). Larvae at 9 DPH were fed several enriched rotifer diets: two microalga concentrates [*Isochrysis* sp. (ISO) and *Pavlova* sp. (PAV)], a semi-moist paste [Protein Selco Plus (PS+)], a combination of the three (Combined), and three dry enrichment diets [AlgaMac 3050 (AM), Ori-Green (OG), and Protein highly unsaturated fatty acids (HUFA; PH); Cavalin and Weirich, 2009]. Larvae at 17 DPH were fed three microdiets [Gemma, Otohime, and a reference diet (LR803); Hauville et al., 2014]. The dashed lines (ratio = 1) indicate equal amounts of fatty acids in the larvae and in the diet. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

FD Ratio in Juveniles

Only a few papers (Rombenso et al., 2016, 2017; Jackson et al., 2020) have discussed the physiological needs of Florida pompano juveniles in terms of fatty acids. In these studies, docosapentaenoic acid (DPA) was also observed in juvenile tissues. We have included it in this review since it has been identified as a potential EFA (Parrish et al., 2007). Rombenso et al. (2016) assessed the fatty acid composition in different tissues of Florida pompano juveniles (43.4 ± 0.2 g) following fish oil replacement (**Table 4**; **Figure 3**). Juveniles were fed six different diets for 8 weeks; the diets contained menhaden fish oil, 25:75 blends of fish oil and standard soybean oil, MUFA-enriched soybean oil, SFA-enriched soybean oil, palm oil, or poultry fat. ARA was selectively retained in the brain irrespective of the diet that was provided (FD ratio $\geq$ 1.35), suggesting a physiological need for this EFA in this tissue. However, the same FA was present at satisfactory levels in the eye, with FD ratios not exceeding 1.14 for all diets (**Figure 3**). The same tendency was observed in the liver except for juveniles fed the palm diet (ratio = 1.57). ARA ratios were equal to zero in the muscle tissue of all juveniles, suggesting that ARA is not required in muscle tissue at this stage and is probably not used for energy purposes (**Figure 3**). On the other hand, DHA and DPA seemed to have been strongly retained in all tissues, with higher selective retention in the brain and eye. FD ratios for DHA were > 2.2 in the eyes of juveniles fed the SFA soy and MUFA soy diets. The FD ratio in brain tissue was as high as 3.28 for the same diets. Higher DPA retention levels were also observed in the eye, with FD ratios $\geq$ 2.22 in juveniles fed all diets, suggesting the importance of this FA in eye development. FD ratios for EPA were $\leq$ 0.5 in all tissues for all diet treatments, which suggests that diets with EPA levels below 3% of total FA (**Table 4**) fulfilled EFA needs at this developmental stage. Other PUFA, such as LA and LNA, showed different trends in different tissues. FD ratios for LA were < 1 in all tissues except for muscle tissue in juveniles fed the fish diet; these had an FD ratio equal to 1, suggesting that this FA might be used for energy purposes. There is little or no physiological need for LNA, as evidenced by the lack of retention in any tissue (ratio < 0) combined with levels below 2% in muscle and eye and 0% in liver and brain (**Table 4**). We suggest that LNA was used mostly as an energy substrate. FD ratios for SFA, MUFA, and PUFA were all close to one in each studied tissue (**Figure 3**).

Rombenso et al. (2017) also evaluated the requirements for EPA and DHA in Florida pompano juveniles (41.0 ± 0.5 g) by feeding them for 8 weeks with diets containing fish oil, beef tallow, or beef tallow partially or fully supplemented with EPA, DHA, or both. It was interesting to see that ARA was more balanced in the brain tissue for fish fed beef tallow supplemented with EPA and DHA (FD ratio = 1.14), while this same fatty acid was more balanced in the eye tissue with all diets, but not with the non-supplemented beef tallow diet (**Figure 4**). ARA was highly retained in liver and muscle tissue except for two diets (fish oil only and the fully supplemented beef diet), where FD ratios were equal to 0.92. DHA and DPA were still highly retained in all tissues. However, DHA and DPA FD ratios were 1.1 and 1.28, respectively, in the brain of juveniles fed the fully supplemented beef diet (**Figure 4**), highlighting these specific needs in the brain tissue. The FD ratio for DHA was 1.04 in the eye tissue of juveniles fed the fully supplemented beef diet, which might suggest that this diet best fulfilled the DHA requirements of Florida pompano juveniles. EPA ratios in all tissues were comparable to a previous

TABLE 3 | Relative percentages of neutral and polar fatty acids in winter flounder larvae (*P. americanus*) at mouth opening, live-prey feeding, and weaning (4; 15–16, 22; and 26 DPH, respectively; Seychelles et al., 2011; Martinez-Silva et al., 2018).

	Larvae			
	Mouth opening	**Live-prey feeding**		**Weaning**
Neutral fatty acid	4 DPH	15–16 DPH	22 DPH	26 DPH
18: 2 n-6 (LA)	4.55 ± 0.78	3.16 ± 1.27	1.39 ± 0.06	5.30 ± 0.42
18: 3 n-3 (LNA)	0	0.20 ± 0.34	0.34 ± 0.12	0
20: 4 n-6 (ARA)	2.45 ± 0.07	2.32 ± 1.18	1.29 ± 0.30	3.55 ± 0.35
20: 5 n-3 (EPA)	9.7 ± 1.1	6.5 ± 3.2	2.9 ± 0.8	3.6 ± 1.3
22:6 n-3 (DHA)	20.25 ± 0.35	7.86 ± 4.81	2.73 ± 0.81	7.30 ± 2.12
Total n-3	31.80 ± 1.13	17.96 ± 9.63	8.19 ± 1.85	19.75 ± 3.32
Total n-6	7.10 ± 0.85	5.82 ± 1.83	3.26 ± 0.23	9.15 ± 0.92
SFA	29.60 ± 3.90	65.44 ± 13.63	74.41 ± 16.00	24.44 ± 0.59
MUFA	27.43 ± 3.51	22.57 ± 9.02	13.2 ± 8.0	36.83 ± 8.86
PUFA	42.97 ± 1.59	11.99 ± 5.92	12.38 ± 8.34	38.73 ± 8.28
Polar fatty acid				
18: 2 n-6 (LA)	3.50 ± 0.00	4.15 ± 0.66	2.30 ± 0.20	4.90 ± 0.71
18: 3 n-3 (LNA)	0	0	0.40 ± 0.01	0
20: 4 n-6 (ARA)	3.55 ± 0.07	5.50 ± 1.68	2.90 ± 0.01	6.10 ± 0.57
20: 5 n-3 (EPA)	11.55 ± 0.21	8.75 ± 4.92	17.26 ± 0.16	9.55 ± 0.49
22:6 n-3 (DHA)	22.90 ± 0.57	13.75 ± 3.48	6.72 ± 0.27	12.35 ± 4.74
Total n-3	35.85 ± 0.78	27.09 ± 1.60	27.16 ± 0.48	28.1 ± 4.9
Total n-6	7.30 ± 0.14	10.15 ± 2.28	6.00 ± 0.23	11.35 ± 0.21
SFA	34.33 ± 0.78	43.48 ± 11.42	52.45 ± 14.50	29.97 ± 1.76
MUFA	20.03 ± 0.76	20.96 ± 3.76	13.75 ± 2.76	23.77 ± 6.23
PUFA	45.57 ± 1.07	35.56 ± 9.95	33.81 ± 12.60	46.23 ± 4.71

Data represent mean ± SD from all diet treatments. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

study conducted by the same authors (Rombenso et al., 2016); FD ratios $\leq$ 0.5. LNA were only found in eye tissue and were zero in all other tissues (**Figure 4**), confirming that there was no specific physiological need for this FA at this developmental stage. FD ratios for LA were close to one in muscle, liver, and eye tissues for all diets tested, indicating an adequate supply of this FA. LA was not selectively retained in the brain tissue, which confirms that it is not required for brain development/function. In all tissues examined, FD ratios for SFA, MUFA, and PUFA were close to one (**Figure 4**), suggesting that most diets tested, except the beef diet, were suitable fatty acid sources for Florida pompano juveniles. Juveniles fed the beef-only diet showed selective PUFA retention in almost all tissues, suggesting that this diet did not provide enough omega n-3 and PUFA to allow proper juvenile development. Thus, diets for juvenile Florida pompano development should include around 2% ARA, 3% EPA, 2% DPA, and 15% DHA, for a total PUFA below 30%, to sustain development.

Winter flounder juveniles showed low EFA accumulation in their neutral lipids until 60 DPS (**Table 5**), and no active retention in lipid reserves was observed except for ARA. Before 60 DPS, all EFA seemed to be directly incorporated into polar lipids, possibly in response to vital physiological requirements. Following settlement, DHA and EPA requirements seemed to be adequately met with the Gemma micro diet and diets of rotifers enriched with commercial Selco DHA protein or with INVE Selco sparkle in older fish, since the FD ratios of these EFA in the lipid polar fraction were below one (**Figure 5**). However, the FD ratio for ARA in the polar fraction was much greater than one in all diets (**Figure 5**), suggesting inappropriate enrichment for this FA. As juveniles grew, the relative content of fatty acids increased except for ARA, which remained close to values observed at the early juvenile stage (**Table 5**). Indeed, almost all FD ratios were below one in young juveniles (60 and 90 DPS), indicating that needs were met regardless of the diet, even though ARA was still actively retained (**Figure 5**). Thus, these results suggest that diets with 10% EPA, 12% DHA, more than 5% ARA, and a total of 40% PUFA could be adequate to sustain proper development in winter flounder juveniles.

QUALITATIVE ESSENTIAL FATTY ACID REQUIREMENTS FOR EARLY STAGES

The qualitative requirements for EFA in Florida pompano larvae were the highest during the live-feeding period, when the relative percentages of DHA, EPA, and ARA represented 16, 7, and 4% of total fatty acids, respectively (**Figure 6**). These findings are comparable to those obtained, at 8 DPH, in a closely related species; the golden pompano (*Trachinotus ovatus*). The authors suggested that at least 18% DHA, 5% EPA, and 3.5% ARA of total FAs are needed for the larvae to achieve optimal development and reduce deformities (Fu et al., 2021). During the weaning period, the requirements for DHA and ARA decreased by 50 and 75% from the live-feeding period, respectively (7 and 1% of total FA for DHA and ARA, respectively), while EPA percentages did not change (7% of

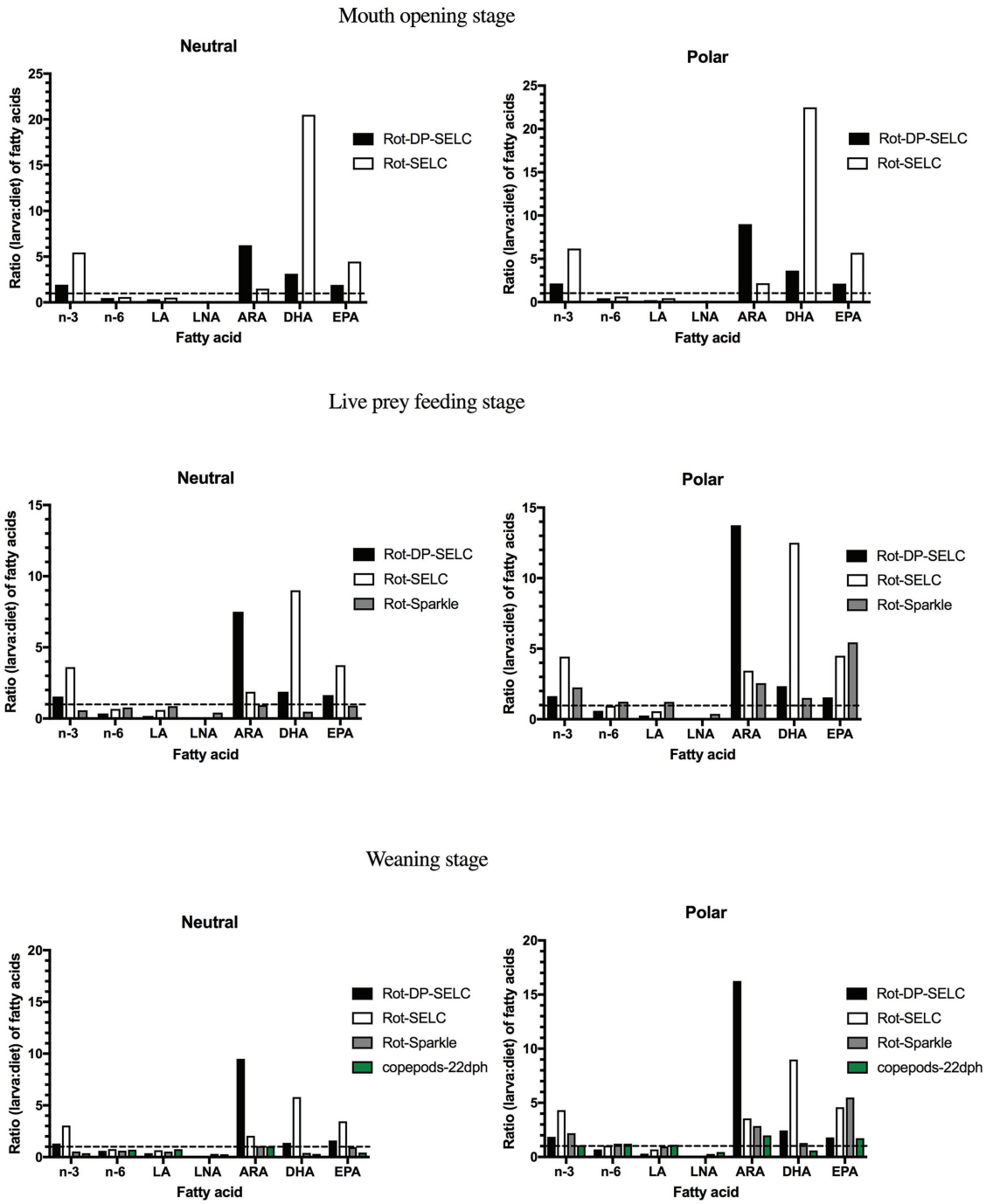

FIGURE 2 | Ratio (larva: diet) of neutral and polar fatty acids in winter flounder (*Pseudopleuronectes americanus*) larvae at mouth opening (4 DPH), live feeding (15–16 DPH), and beginning of weaning (26 DPH). Larvae had been fed several enriched rotifer diets: Selco 3000 (SELC), DHA Protein Selco (DP-SELC), and sparkle-INVE (Sparkle). Larvae at 22 DPH were fed copepods (Seychelles et al., 2011; Martinez-Silva et al., 2018). The dashed lines (ratio = 1) indicate equal amounts of fatty acids in larvae and in the diet. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; and EPA, eicosapentaenoic acid.

total FA; **Figure 6**). The relative percentages of LA and LNA increased over time, from 3 to 5% of total FA for LA and from 0 to 1% of total FA for LNA. By comparison golden pompano larvae that were fed several enriched *Artemia nauplii* diets from 11 to 27 DPH, showed a slightly lower requirement for DHA (5% of total FAs), but relatively similar requirements for EPA, ARA, LA, and LNA (6.7, 1.3, 3, and 1% of total FAs, respectively; Ma et al., 2016).

TABLE 4 | Relative percentages of fatty acids in juvenile Florida pompano (*T. carolinus*; 43.4 ± 0.2 g) muscle, liver, eye, and brain tissues.

Fatty acid	Muscle	Liver	Eye	Brain
18: 2 n-6 (LA)	7.41 ± 4.19	5.25 ± 2.80	7.18 ± 3.95	1.74 ± 1.22
18: 3 n-3 (LNA)	1.27 ± 0.57	–	0.76 ± 0.18	–
20: 4 n-6 (ARA)	1.09 ± 0.17	1.93 ± 0.94	0.91 ± 0.19	1.48 ± 0.16
20: 5 n-3 (EPA)	3.21 ± 1.19	1.46 ± 1.07	3.44 ± 1.63	1.59 ± 0.30
22:5 (DPA)	1.71 ± 0.60	1.69 ± 0.82	2.47 ± 0.67	1.29 ± 0.27
22:6 n-3 (DHA)	10.80 ± 3.09	15.22 ± 5.14	11.43 ± 1.96	14.98 ± 2.68
SFA	38.09 ± 2.77	40.33 ± 2.54	38.82 ± 2.76	47.84 ± 2.07
MUFA	35.34 ± 4.22	31.91 ± 6.80	33.36 ± 5.21	30.61 ± 1.53
PUFA	26.52 ± 4.16	27.69 ± 7.01	27.80 ± 4.79	21.54 ± 3.01

Juveniles were fed six diets for 8 weeks. Diets contained menhaden fish oil or 25:75 blends of fish oil and standard soybean oil, MUFA-enriched soybean oil, SFA-enriched soybean oil, palm oil, or poultry fat. Data represent mean ± SD from all diet treatments. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

The same tendency was observed for winter flounder larvae, with decreased EFA requirements over time. However, EFA requirements at all winter flounder larva stages were higher than for Florida pompano larvae. The relative percentages of DHA, EPA, and ARA were 23, 12, and 4% of total FA at the mouth-opening stage (**Figure 6**). The relative percentage of DHA decreased by 75% at the live-prey feeding and weaning stages (8 and 7% of total FAs, respectively; **Figure 6**), while the relative percentages of EPA decreased slightly to 9% of total FA at the live-prey feeding stage and increased to 17% of total FA at the weaning stage (i.e., 26 DPH). The relative percentages of ARA decreased by 1% at both the live-prey feeding and weaning stages. LA requirements were the same during the first days of life and decreased by half (2% of total FA) at the weaning stage (**Figure 6**).

Overall, winter flounder larvae had higher requirements for omega 3 (n-3) FA at the mouth-opening stage (36% of total FA) than at the live-prey feeding and weaning stages (28–27% of total FA), while requirements for omega 6 (n-6) seemed to increase over time (7% of total FA at the mouth-opening stage vs. 11–12% of total FA at the live-prey feeding and weaning stages).

Studies on other marine finfish species have shown that the dietary inclusion of n-3 HUFA (i.e., DHA and EPA) and n-6 HUFA (i.e., ARA) improved larval growth, development, and metamorphosis. When examining additional nutritional studies on warm-water and cold-water marine finfish species, we found that EFA requirements during larval development from first feeding to weaning were similar, with few exceptions, to the results reported here. Indeed, common snook (*Centropomus indecimalis*) larvae at the rotifer-feeding stage (live-prey feeding) showed the same qualitative requirements for EFA as Florida pompano (15, 7, and 3% of total FA for DHA, EPA, and ARA, respectively; Hauville et al., 2016). Red drum (*Sciaenops ocellatus*) larvae at the weaning period (18 DPH) were found to have slightly lower requirements for DHA and EPA (4 and 5% of total FA, respectively) and similar requirements for ARA (1% of total FA) compared to Florida pompano larvae at the same developmental stage (Brinkmeyer and Holt, 1998).

These requirements seemed to be lower in warm-water marine fish at the weaning period, particularly for DHA and ARA, suggesting that the EFA requirements are higher in rapidly growing larvae at the live-feeding stage. In addition, the ratio of DHA to EPA is greater at the live-feeding period (2.2) than during the weaning period (1.0; **Figure 6**), which is similar to what was observed in red drum larvae as well as larvae from four other species of marine finfish (Watanabe and Kiron, 1995; Brinkmeyer and Holt, 1998). It has been suggested that the high DHA to EPA ratios at the live-feeding stage play a role in stress resistance. The high levels of DHA observed during early larval development for both species reflect the importance of this EFA in larval structural development and in neural and visual functions (Bell et al., 1995a,b; Sargent et al., 1999).

The qualitative requirements for DHA and ARA appeared to be higher in larvae of species that undergo metamorphosis. DHA and ARA values reported in southern flounder (*Paralichthys lethostigma*) and Atlantic halibut (*Hippoglossus hippoglossus*) larvae at 15 DPH (live-prey feeding period) were 17 and 5% of total FA, respectively (Hamre and Harboe, 2008; Alam et al., 2015; Oberg and Fuiman, 2015; Hamre et al., 2020), which is more than twice the percentage of DHA and five times the percentage of ARA found in Florida pompano larvae at the same developmental stage. These differences strongly suggest greater requirements for DHA and ARA during the physiologically demanding metamorphosis process. Hamre et al. (2020) suggested that FA needed to have at least 13% DHA for normal pigmentation in Atlantic halibut, highlighting the effect of DHA both on eicosanoid production and the development of vision and nervous tissue (Denkins et al., 2005; Roman et al., 2007; Hamre et al., 2020).

An adequate EPA to ARA ratio is important to achieve normal pigmentation and complete eye migration in flatfish species; this has been reported for turbot (*Scophthalmus maximus*), Atlantic halibut, Senegalese sole (*Solea senegalensis*), and yellowtail flounder (*Limanda ferruginea*; McEvoy et al., 1998; Estévez et al., 1999; Copeman et al., 2002; Villalta et al., 2005). The EPA to ARA ratio in winter flounder was 3.0 during early larval development (4 and 15 DPH) and close to 6.0 at the weaning period (22–26 DPH; **Figure 6**). For Florida pompano, the highest ratio (ratio = 7.0) was reported at the weaning period, but other than that, the ratio did not exceed two. A previous study on turbot larvae has concluded that it is important to consider the ratio of DHA to EPA and found that it is positively correlated with pigmentation success (Rainuzzo et al., 1994).

At the juvenile stage, the qualitative requirement for DHA was similar between the two species (12% of total FA). However, 0+ juvenile winter flounder were much smaller than juvenile Florida pompano (~ 1 vs. 42 g), thus their DHA requirement might be higher for the same stage of development (**Figure 6**). The different DHA requirements can also be coupled with water temperatures of the different habitats, with cold water fish species generally having higher DHA levels than more temperate species (Hamre et al., 2020). In general, poikilotherm fish counteract the lower temperature effect to maintain their

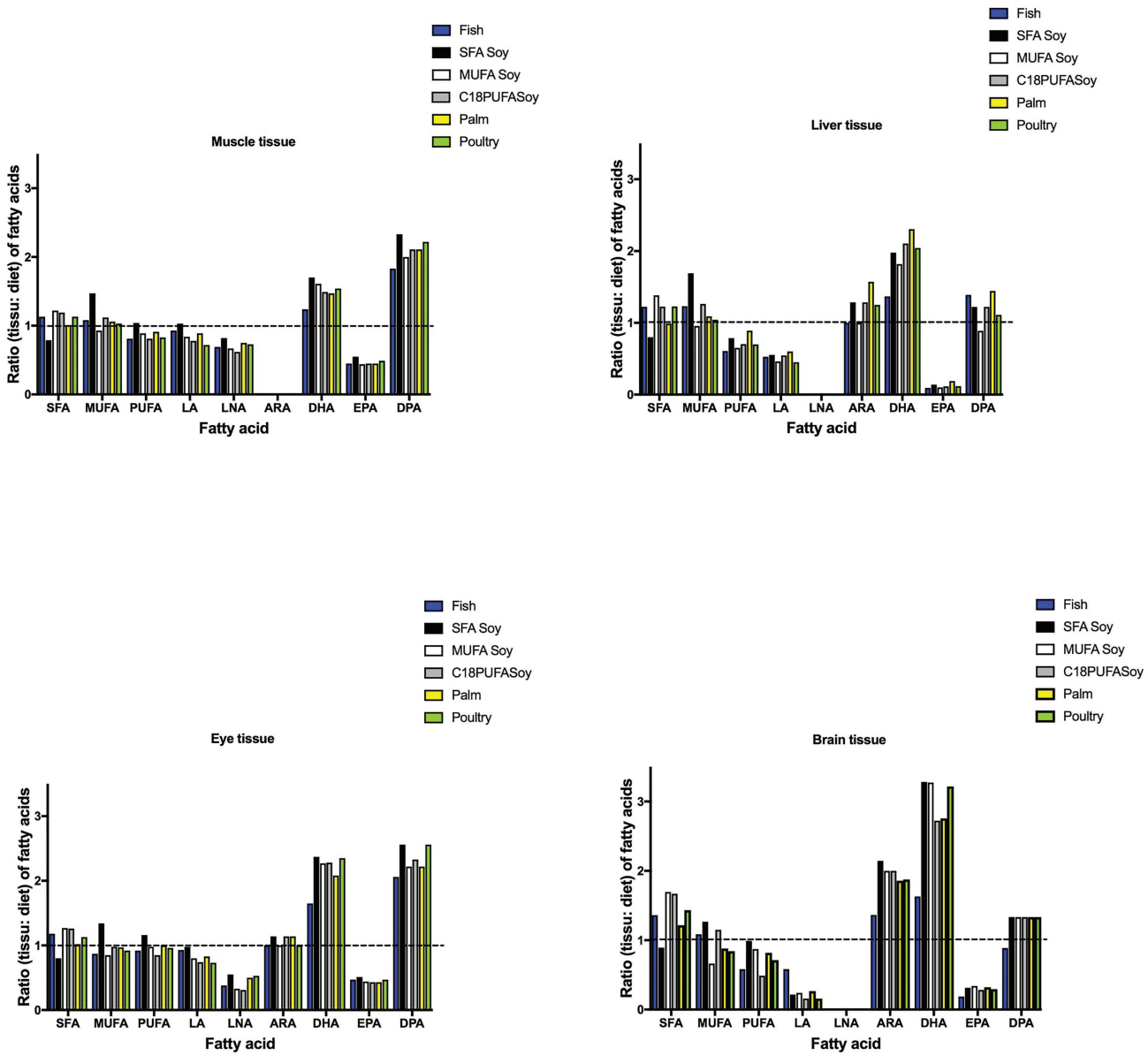

FIGURE 3 | Ratio (tissue: diet) of fatty acids in juvenile Florida pompano (*T. carolinus*) muscle, liver, eye, and brain tissue (44 g average weight). Diet treatments consisted of a diet containing menhaden fish oil (Fish), SFA-enriched soybean oil (SFA Soy), MUFA-enriched soybean oil (MUFA Soy), 25:75 blends of fish oil and standard soybean oil (C18 PUFA Soy), palm oil (Palm), or poultry fat (Poultry; Rombenso et al., 2016). The dashed lines (ratio = 1) indicate equal amounts of fatty acids in the juveniles and in the diet. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

metabolism by remodeling fatty acids in the membrane. In cold conditions, the integration of PUFA, particularly DHA, in phospholipids maintains membrane fluidity and metabolic rate, a process known as homeoviscous adaptation (Hazel, 1995). Thus, cold-water species should require more DHA to maintain their physiological function, as observed in this comparison between winter flounder and Florida pompano. EPA and ARA requirements seemed to be higher in winter flounder, ranging from 6% at the early settlement stage to 11% at the 0+ juvenile stage for EPA and from 3 to 4% for ARA for the same stages, respectively (EPA: ARA ratio is approximately 2.5; **Figure 6**). Hamre et al. (2020) showed that Atlantic halibut juveniles would need a combination of at least 13% DHA of total FA and a higher ratio of EPA to ARA (ratio = 3.5) to promote growth and survival of young juveniles.

One of the most interesting differences between the two species was the presence of LA and LNA in Florida pompano larvae and juveniles at all stages, while LNA was absent in winter flounder. Jackson et al. (2020) recently published data suggesting that Florida pompano juveniles have some

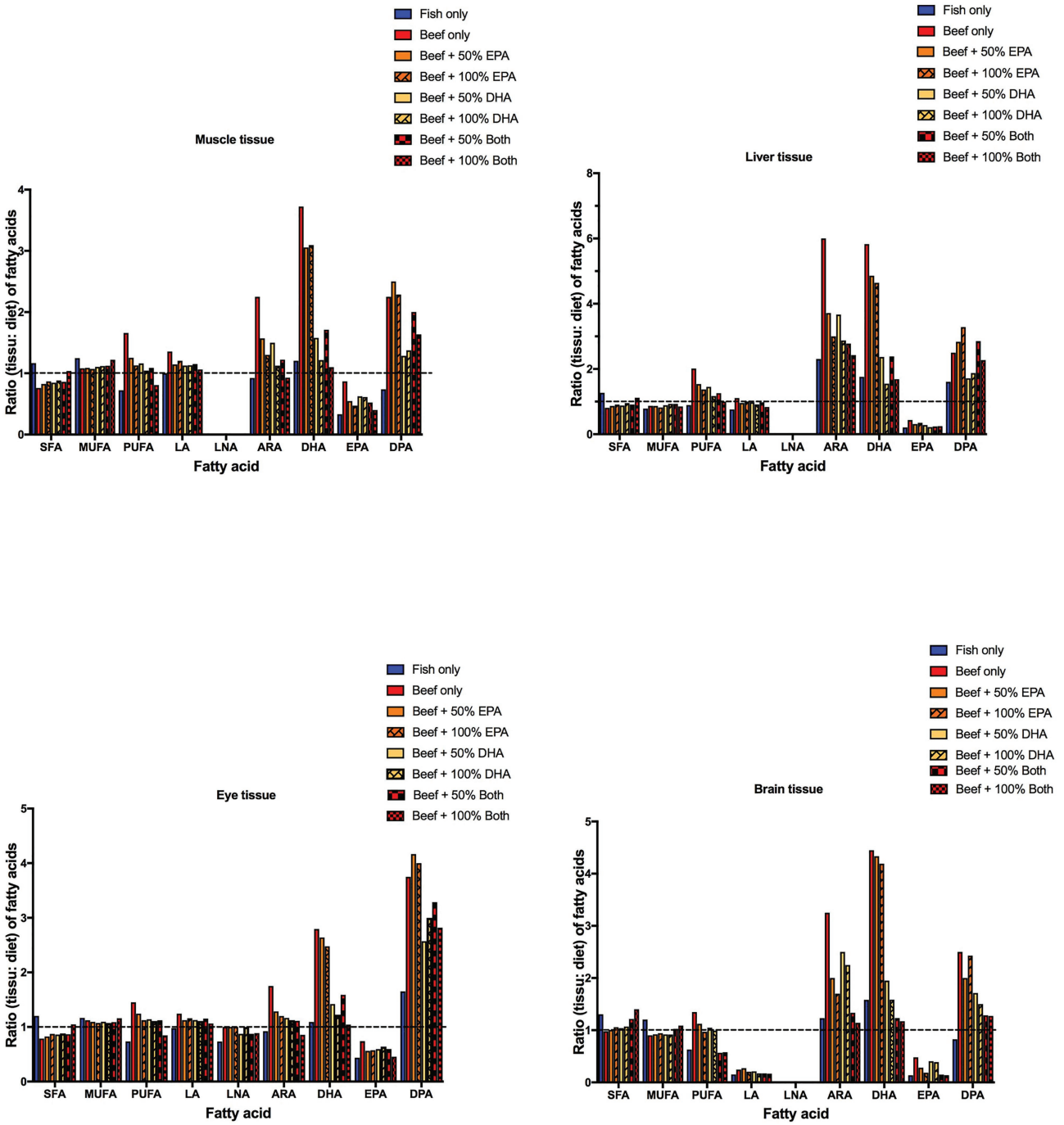

FIGURE 4 | Ratio (tissue: diet) of fatty acid in juvenile Florida pompano (*T. carolinus*) muscle, liver, eye, and brain tissues (41 g average weight). Diet treatments contained fish oil, beef tallow, or beef tallow partially or fully supplemented with EPA, DHA, or both (Rombenso et al., 2017). The dashed lines (ratio = 1) indicate equal amounts of fatty acids in juveniles and in the diet. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

capacity to elongate and desaturate fatty acids from C18 precursors (i.e., LA and LNA) and may be able to survive on diets containing C18 PUFA. However, the authors strongly recommend that juveniles be directly provided with HUFA to perform optimally (Jackson et al., 2020). Recent studies concluded that golden pompano juveniles (ranging in sizes from 8 to 50 g), a species belonging to the same family as the Florida pompano, might have low capacity to biosynthesize HUFA (Liu et al., 2018; Li et al., 2020; Wang et al., 2020). Wang et al. (2020) have found that golden pompano juveniles may have the capability of converting EPA to DHA but lack the Δ5 desaturation activity, required

TABLE 5 | Relative percentages of neutral and polar fatty acids in winter flounder (*P. americanus*) juveniles at the beginning of settlement (38 DPH), 30 DPS, and at the 0+ juvenile stage (60 and 90 DPS).

	Juveniles				Reference
	Settlement	Early settled juveniles	0+juveniles		
Neutral fatty acid	38 DPH	30 DPS	60 DPS	90 DPS	
18: 2 n-6 (LA)	5.40 ± 0.00	5.55 ± 0.07	7.27	7.60	38 DPH and 30 DPS: Seychelles et al., 2011, and Bélanger et al., 2018
18: 3 n-3 (LNA)	0	0	0	0	60 and 90 DPS: Bélanger et al., 2018
20: 4 n-6 (ARA)	1.65 ± 0.92	1.10 ± 0.14	3.15	1.53	
20: 5 n-3 (EPA)	2.50 ± 0.0	2.40 ± 0.14	9.01	6.90	
22:6 n-3 (DHA)	1.00 ± 0.00	1.00 ± 0.00	10.57	6.80	
Total n-3	4.10 ± 0.14	3.70 ± 0.00	21.01	14.44	
Total n-6	7.05 ± 0.92	6.65 ± 0.07	16.27	15.68	
SFA				28.00	Bélanger et al., 2018
MUFA				34.05	
PUFA				34.95	
Polar fatty acid					
18: 2 n-6 (LA)	7.20 ± 0.28	7.85 ± 0.07	5.19	7.02	38 DPH and 30 DPS: Seychelles et al., 2011, and Bélanger et al., 2018
18: 3 n-3 (LNA)	0	0	0	0	60 and 90 DPS: Bélanger et al., 2018
20: 4 n-6 (ARA)	3.30 ± 0.00	3.00 ± 0.00	4.60	4.03	
20: 5 n-3 (EPA)	6.50 ± 0.00	6.00 ± 0.00	10.88	10.88	
22:6 n-3 (DHA)	3.75 ± 1.77	2.50 ± 0.71	12.53	10.81	
Total n-3	12.45 ± 1.63	6.95 ± 3.61	23.99	22.56	
Total n-6	10.65 ± 0.35	14.0 ± 4.24	13.99	15.79	
SFA				33.35	Bélanger et al., 2018
MUFA				23.55	
PUFA				41.60	

Data represent mean ± SD from all diet treatments. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

to convert 20:3 n-6 and 20:4 n-3 to ARA and EPA, respectively, suggesting incomplete HUFA biosynthesis ability.

It is hypothesized that trophic level is a better predictor of a requirement for C18 PUFA vs. HUFA rather than the species' thermal or salinity preference (Jackson et al., 2020; Trushenski and Rombenso, 2020). It is interesting to note that Florida pompano and winter flounder, despite their contrasting thermal environments, both belong to the same trophic level (Florida pompano trophic level = 3.5, Froese and Pauly, 2016; winter flounder trophic level = 3.6 ± 0.1, Murdy et al., 1997), which might explain some of the similarities in EFA requirements observed here. The validation of this hypothesis would change our way of investigating requirements for fatty acids in fish species and thus allow standardization of feed formulations for different developmental stages.

CONCLUSION

Essential fatty acid requirements vary qualitatively and quantitatively during fish ontogeny, with the larval and juvenile stages being arguably the most critical periods. In this review, we give a comprehensive synthesis of these requirements, highlighting the similarities and differences between two species occupying contrasting environments: a warm-water tropical species (Florida pompano) and a cold-water flatfish species (winter flounder). In general, we found that the young larvae of both species are characterized by greater requirements for n-3 and n-6 HUFA compared to larvae at the live-feeding and weaning stages as well as juveniles. Florida pompano larvae at the live-feeding stage require more DHA, EPA, and ARA than larvae at the weaning stage, with minimal levels probably around 15, 7, and 3.5% of total FA, respectively, for young larvae (i.e., 9 DPH) and 9, 5, and 0.5% of total FA for larvae at the weaning stage. Nevertheless, none of the diets seemed to fully satisfy the EFA needs for both larval stages. Studies on the EFA requirements during early larval development in winter flounder suggest that the physiological needs of larvae were not met for all the rotifer-enriched diets tested. Ratios of DHA, EPA, and ARA in the polar lipid fraction related to diet were systematically well over 1.0, indicating strong retention. Ratios ≤ 1 were only obtained when larvae at the weaning stage were fed copepods, suggesting a minimal qualitative requirement of 12% DHA, 10% EPA, and 6% ARA of total FA at this stage, and up to 20% DHA in younger larvae. During metamorphosis, winter flounder – like many flatfish species – has specific EFA requirements necessary to achieve correct pigmentation and eye migration. Thus, an early supply of dietary DHA, EPA, and ARA emphasizing the importance of dietary DHA: EPA: ARA ratios is essential for successful pigmentation and eye migration. Concerning juvenile development, the qualitative EFA requirements seem to be similar in both species, with slightly higher needs for EPA and ARA in winter flounder. Diets containing around 15% DHA, 3% EPA, 2% DPA, and 2% ARA, for a total PUFA below 30% of total FA, seem to be appropriate for Florida pompano

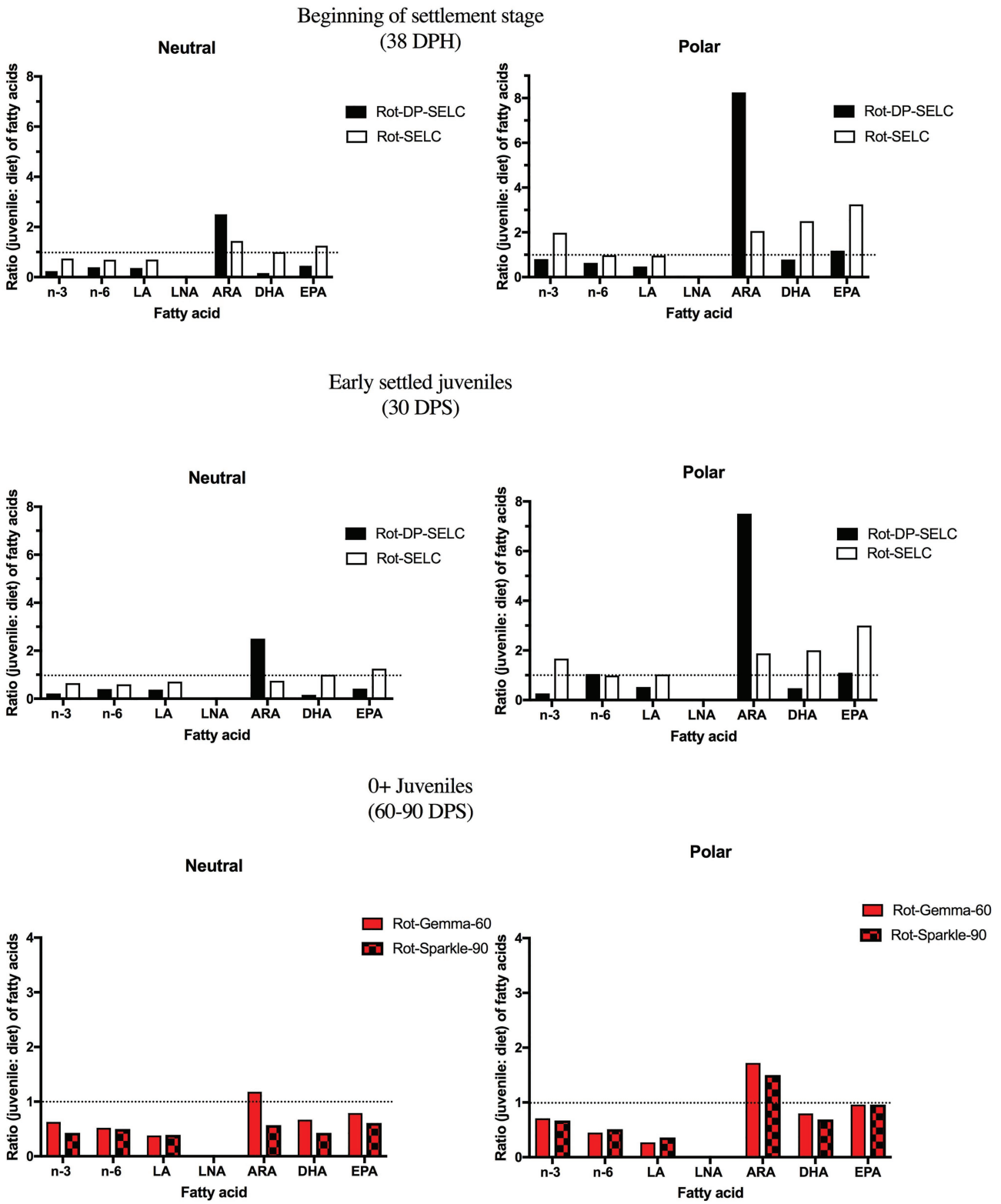

FIGURE 5 | Ratio of neutral or polar fatty acids in winter flounder (*P. americanus*) juveniles relative to diet total fatty acids contents (juvenile: diet) at the beginning of settlement (38 DPH), early settled juveniles [30 days post settlement (DPS)], and 0+ juveniles (60–90 DPS) fed several enriched rotifer diets: Selco 3000 (Rot-SELC), DHA Protein Selco (DP-SELC), sparkle-INVE (Rot-Sparkle), and Gemma microdiet (Rot+Gemma). The dashed lines (ratio = 1) indicate equal amounts of fatty acids in the juveniles and in the diet. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; and EPA, eicosapentaenoic acid.

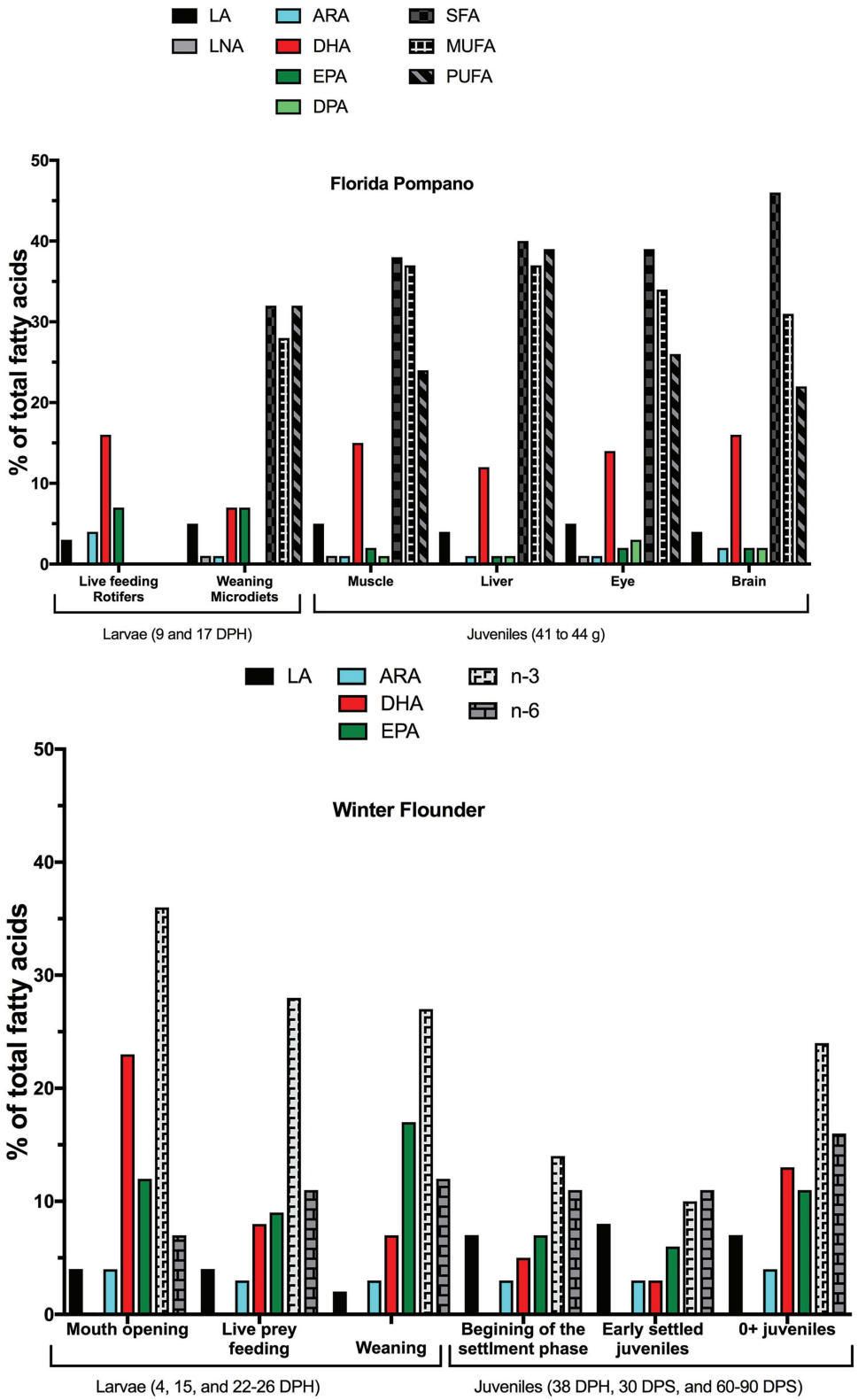

FIGURE 6 | Optimum relative percentages of essential fatty acids (EFA) in Florida pompano (*T. carolinus*) larvae and juveniles (top) and winter flounder (*P. americanus*) larvae and juveniles (bottom). We determined the optimum qualitative requirements (percentages) from data that had larva to diet or juvenile to diet ratios closest to one. DPH, days post hatch; DPS, days post settlement; LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

development, while diets with 12% DHA, 10% EPA, and 5% ARA, for a total of 40% PUFA, could be better for juvenile winter flounder development.

Identifying the EFA needs in marine fish larvae is particularly difficult because of the fishes' small size and often poorly developed digestive system. The complexity and time-consuming effort of determining precise microdiets led to the use of live feeds, such as rotifers and *Artemia*, but these are often inadequate for marine larvae and require enrichment in HUFA. HUFA enrichments of live feeds do not provide sufficient DHA and adequately balanced levels of HUFA (Tocher, 2010), thus it is necessary to continue work toward developing nutritionally balanced microdiets that satisfy the physiological needs of marine fish larvae. In addition, the growing aquaculture industry – by pressing the need for marine fish meal and oils to be replaced with plant-derived products – has reintroduced the need to identify the precise qualitative and quantitative EFA requirements for marine fish larvae and juveniles. There must be detailed knowledge of the molecular and biochemical bases of HUFA requirements and metabolism: the physiological needs of a fish species to achieve optimal growth and stay healthy are different from the requirements to maintain nutritional quality, which leads to health benefits for human consumers.

AUTHOR CONTRIBUTIONS

SM and RT have developed the idea and all authors contributed to this review. All authors contributed to the article and approved the submitted version.

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REGULAR PAPER

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A comprehensive biochemical characterization of settlement stage leptocephalus larvae of bonefish (*Albula vulpes*)

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ABSTRACT

Little is known about early development of the near-threatened bonefish (*Albula vulpes*), a member of superorder Elopomorpha. Members of Elopomorpha are partially defined by their synapomorphic leptocephalus larval stage, for which the nutritional requirements are not well understood. Characterizing the nutritional profile, including major nutrients (such as lipids) used for energetic processes, can help to gain a better understanding of the nutritional requirements for leptocephalus larvae. A total of 24 settlement stage *A. vulpes* leptocephalus larvae were collected at Long Caye Island, Belize. Samples were used to determine various biochemical characteristics including lipid class, fatty acid and glycosaminoglycan compositions. Each of these biochemical components plays a role in early developmental processes such as cellular membrane formation and is crucial for healthy development. Biochemical characteristics of settlement stage *A. vulpes* leptocephalus are presented in this study for the first time. The dominant lipid classes and fatty acids detected in these samples were consistent with prior studies using closely related species like the Japanese eel, indicating possible similarities in diets at this stage. In the future, similar analyses can be applied to other species that share the leptocephalus life stage to determine if nutritional requirements at this stage of development are unique to this species. The findings in this study will also help to facilitate the establishment of adequate aquaculture systems for captive bonefish, ultimately leading to improved management strategies for wild bonefish habitats.

KEYWORDS
fatty acids, glycosaminoglycans (GAGs), larval nutrition, leptocephalus, lipid classes

1 | INTRODUCTION

Bonefish (*Albula* spp.) are one of the oldest extant members of the order Elopomorpha, which includes eels, ladyfish and tarpon. The primary evolutionary synapomorphy that unites the order is a unique larval stage called the leptocephalus ("slender head"). Leptocephali are transparent, gelatinous, ribbon-shaped larvae with bodies composed largely of acellular glycosaminoglycan (Bishop *et al*., 2000; Pfeiler, 1991) that can reside in the water column for extended periods of time. *Albula vulpes* larvae in the Bahamas have a mean larval duration of 54 days, with durations ranging from 41 to 72 days (Mojica, 1995). This finding is similar to the 56 day mean larval duration of other bonefish species (*A. glossodonta*) native to the Pacific Ocean (Friedlander, 2007). The absence of recognizable prey in their intestines and their unusual fang-like dentition has inspired numerous hypotheses about their mode of feeding and how they acquire nutrients for metabolism and growth.

Known to recreational anglers as the "grey ghost", bonefish carry historical, recreational and cultural significance in countries where

they occur. Bonefish are typically found in shallow-water habitats in tropical regions around the world. The dominant species in the Atlantic Ocean, *Albula vulpes*, is currently listed as a near-threatened species by the International Union on the Conservation of Nature (IUCN) (Adams *et al*., 2012). Throughout the Caribbean and South Florida, bonefish are an economically important species and contribute to thriving recreational fisheries. Management efforts (such as catch and release legislation and water quality monitoring programs) to protect the recreational fishery have been established in many locations, including the Bahamas, Florida (USA) and Belize (Adams *et al*., 2019; Brownscombe *et al*., 2019). Because spawning occurs offshore at night and the leptocephalus larvae are difficult to capture in the open ocean, field-based studies of reproduction and larval biology are very challenging. Therefore, aquaculture of bonefish holds promise for understanding the reproductive and larval biology of the species that can then be used to evaluate potential hypotheses about the biological and ecological factors affecting population dynamics (Adams *et al*., 2019). Recent efforts to induce bonefish spawning from wild broodstock in captivity have been successful and yielded larvae that lived up to 10 days post-hatch (Wills, unpublished data), but lack of understanding of nutrient profiles from wild larvae has limited the lifespan of captive larvae.

Recent research has led to multiple hypotheses on the breeding behaviour of bonefish in the Bahamas and Southern Florida, and assessed the nutritional profiles of multiple wild breeding populations (Lombardo *et al*., 2020; Mejri *et al*., 2018, 2019). Studies regarding the influence of broodstock diets on spawn quality have demonstrated the direct effects of available nutrients in maternal diets on egg and larval survival and development (Mejri *et al*., 2018). Lipid profiles within eggs and larvae are important because at the embryonic and early larval stages, before the onset of exogenous feeding, young bonefish rely on lipid reserves for energetic and metabolic needs (Dahlgren *et al*., 2007; Mejri *et al*., 2018, 2021). In the post-metamorphic settlement stage, leptocephali have limited lipid reserves and begin metabolizing complex structural carbohydrates called glycosaminoglycans (GAGs) as fuel for metabolism and cellular development (Bishop *et al*., 2000; Glencross, 2009). When food is sparse, leptocephali can depend on GAG reserves, making them resistant to starvation.

Recent research on eel leptocephalus larvae have shown that gut diet content correlates with contents found in marine snow such as appendicularian houses and zooplankton faecal pellets (Chow *et al*., 2019; Tsukamoto & Miller, 2021). Given their phylogenetic relationship, it is reasonable to believe that bonefish larvae have a similar diet in the wild. This research also supports the theory that leptocephali utilize GAGs for energy because these marine snow components contain the polysaccharide building blocks of the GAGs found within the mucinous pouch of leptocephalus bodies (Moore *et al*., 2020; Tsukamoto & Miller, 2021). Aquaculture methods for rearing leptocephali have been most successful for captive eels such as the Japanese eel, where diets consisting of a powdered shark egg slurry were most successful in rearing healthy leptocephali (Hamidoghli *et al*., 2019). In the United States, obtaining shark egg yolks is both costly and unsustainable, which has prompted studies like this one to explore bonefish nutritional requirements with the ultimate goal of developing an effective larval rearing diet without shark eggs.

The following is a pioneer study that includes a comprehensive biochemical analysis of post-metamorphic *A. vulpes* leptocephalus samples from Lighthouse Reef Atoll in Belize. By more extensively characterizing this life stage, this research aims to establish a comparable baseline that will improve our understanding of the early leptocephalus nutritional requirements and help to develop appropriate larval diets for captive breeding operations. Successful captive breeding will allow for long-term, in-depth monitoring of bonefish breeding mechanisms and physiological characteristics from hatch to adulthood, information that may become crucial to conservation. In addition, this information will enrich the current data about the larvae of other elopomorph species and further understanding of the bonefish life cycle.

2 | MATERIALS AND METHODS

2.1 | Field sampling and biometrics

A total of 24 *A. vulpes* leptocephalus larvae at settlement stage were collected in a 40 × 60 cm light trap made of 500 μm mesh plankton netting (Sea Gear Corp., Melbourne, FL, USA) with a dive light attached to the centre of the trap, off of the Itza resort pier on Long Caye, Lighthouse Reef Atoll, Belize (17°13′15.85″N, 87°35′26.51″W; Figure 1). The trap was deployed each evening and retrieved in the morning over the course of 1 week (7–14 May 2019). After taking total length measurements, individual larvae were placed in cryovials and frozen at −5°C for a week before shipment to the research laboratory, where they were stored at −80°C prior to analysis. These larvae were then freeze dried to determine water content. Lyophilized larvae were pooled in groups of three to meet the minimum weight requirement for analyses. Of these pools, three were used for glycosaminoglycans analysis and five were used for lipidomic analysis.

Analyses were performed to determine lipid class composition and profiles within each pool of leptocephalus samples. Lipids were isolated from each sample according to methods developed by Folch *et al*. (1957) and modified by Parrish (1999). The resulting extracts from each pool of larvae were spotted in duplicate onto a series of S4 type Chromarods on which they were separated by class using a four-solvent series of thin layer chromatography developments (Parrish, 1987, 1999). Lipid class composition was determined using flame ionization detection techniques via the Iatroscan MK-6s TLC-FID/FPD (Iatron Laboratories Inc. Tokyo, Japan/Shell USA, Spotsylvania, VA, USA). The resulting chromatograms allowed for the identification of the following lipid classes: ketones (KET), triacylglycerols (TAGs), wax esters/steryl esters (WE-SE), free fatty acids (FFA), hydrocarbons (HC), sterols (ST), acetone-mobile polar lipids (AMPL) and phospholipids (PLs). The Iatroscan MK 6s was calibrated using known compound classes from an analytical standard [a mixture of *n*-nonadecane (99%), cholesteryl stearate (96%), 3-hexadecanone (95%), cholesterol (95%) (Thermo Fisher Scientific, Sigma-Aldrich, St. Louis, MO, USA), glyceryl tripalmitate, palmitic acid, l-α-phosphatidylcholine (Sigma-Aldrich, St.

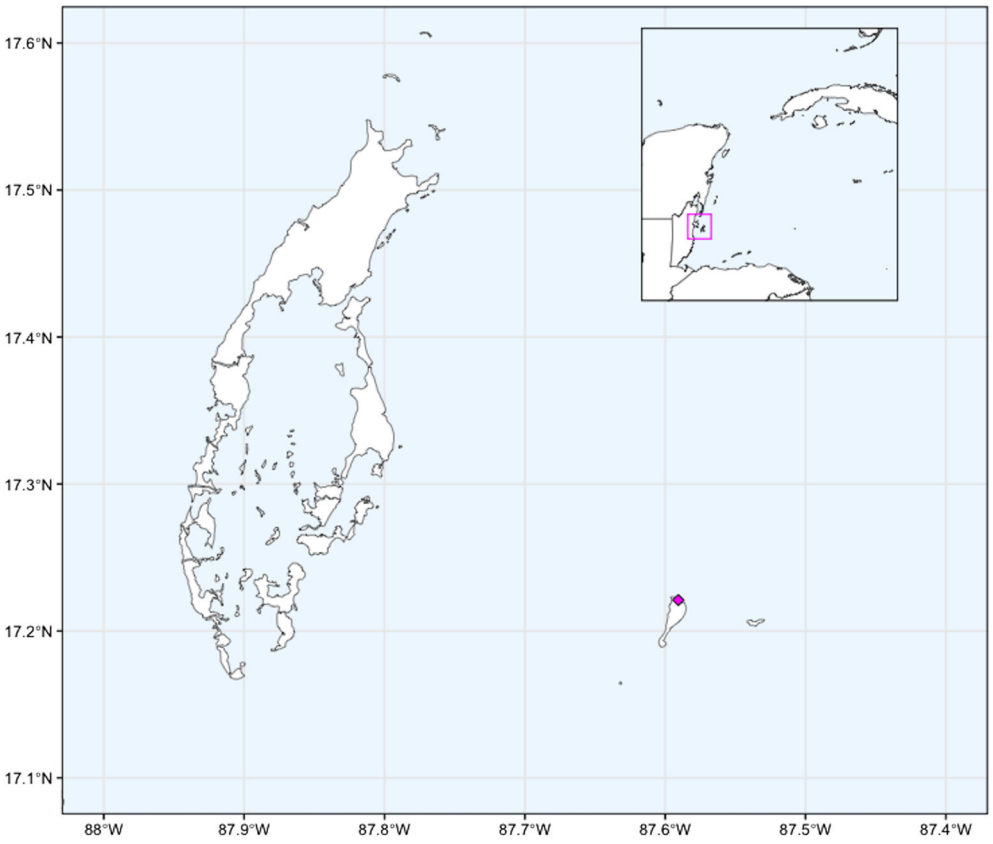

FIGURE 1 Map showing the location of the Long Caye, Lighthouse Reef Atoll, Belize (17°13′15.85″N, 87°35′26.51″W) with the sampling site shown at the diamond marker. The inset map shows a wider view of the Caribbean region with the sampling area within the pink square

Louis, MO, USA), 1-*O*-hexadecyl-*rac*-glycerol (BACHEM America Inc., Torrance, CA, USA)] and peaks were quantified using Peak 453 software. The readings for each lipid class were standardized to mg g^{-1} of lipid extract dry weight (DW) and the total lipid content was calculated by taking the sum of all lipid classes. Relative percentages of each lipid class were calculated by dividing the contribution of individual lipid classes by the total lipid content (mg g^{-1} DW).

2.2 | Fatty acid analysis

The prepared lipid extracts mentioned above were further treated to isolate fatty acids and separated into neutral and polar lipid fractions. The aforementioned lipid extracts were separated using a HyperSep SPE column (Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples were loaded into individual columns, eluted with 10 ml of dichloromethane:methanol (98%:2%) solution to extract neutral lipids and again eluted with 100% methanol to extract polar lipids. Fatty acid methyl esters (FAMEs) were prepared for the gas chromatography/mass spectrometry (GCAQ11-MS) using modified methods described by Lepage and Roy (1984) and suspended in 250 μl of hexane. Methodology was identical for both neutral and polar fraction samples. Fatty acid extracts were analysed with a Clarus 680/600 T GC-MS (Perkin-Elmer, Waltham, MA, USA) using a 30 m Thermo Fisher TR-5 general purpose column with a 250 μm diameter. Each sample was injected into the column (using an 82-vial autosampler) at a volume of 1.0 μl and heated to 250°C where it was held for 10 min. The different fatty acids detected were compared to a 37-component FAME standard (Supelco 37 FAME Mix, Millipore Sigma, Burlington, MA, USA) with known concentrations for quantification purposes. A subsample of the lipid extracts was also used to produce FAMEs for analysis at a second laboratory for comparison and validation of the in-house readings (Creative Proteomics, Shirley, NY, USA).

2.3 | Glycosaminoglycan analysis

A subsample of larvae (three pools of three larvae each) was sent to a partner laboratory (Creative Proteomics Shirley, NY, USA) for GAG analysis. In the pooled samples, GAGs were isolated by first removing lipids and proteins from homogenized samples. Isolated GAGs were then treated with enzymes targeting each of the GAG groups to be quantified (heparinase I-III and chondroitinase ABC) to break down the GAG isolate into disaccharide groups that were detected using strong-anion-exchange-high-performance-liquid-chromatography (SAX-HPLC) (Agilent Technologies Inc., Santa Clara, CA, USA) coupled with fluorescence detection techniques via post-column derivatization, producing chromatograms for each sample. The column used was a 4.6 × 250 mm Waters Spherisorb analytical column with a particle size of 5 μm at 25°C. Each sample was injected at a volume of 10 or 30 μl and the flow rate was set to 1.0 ml min^{-1} with time-dependent solvent gradient settings. Post-column derivatization was achieved by rapidly heating the column eluent to 120°C and immediately cooling in a 50 cm coil, where it was directed into a fluorescence

detector. Disaccharide classes within samples were quantified by comparing elution times to a commercial disaccharide mix, which was also used for calibration. It is important to note that this analysis did not quantify total GAGs, but rather the individual quantities of chondroitin/dermatan sulfate and heparin/heparan sulfate and their relative percentages. This analysis did not quantify the amounts of hyaluronan or keratan sulfate in the samples.

2.4 | Ethics approval

All applicable international, national and/or institutional guidelines for ethical sampling, care and experimental use of organisms for the study have been followed and all necessary approvals have been obtained.

3 | RESULTS

3.1 | Physical characteristics

Leptocephali ranged from 38 to 57 mm total length (TL), with a mean (±S.D.) of 45.11 ± 7.18 mm ($n = 24$). In addition, mean individual wet weight (WW) per larvae was 105.83 mg (S.D. ± 52.62 mg), with a mean water content of 88.55 ± 1.30% of WW ($n = 24$).

3.2 | Lipid class composition

Identifying the classes of lipids present in the leptocephali helps define the compounds that serve as structural components and energy sources for various developmental processes. The mean total lipid content across all sample pools ($n = 8$, three individuals per pool) was 33.75 mg g^{-1} DW (S.D. ± 6.45 mg). The most abundant lipid class in the larvae samples was sterols (ST), followed by PLs, and wax/steryl esters (WE-SE) were least abundant (Table 1). The mean TAG:ST ratio (energy storage:cell structure ratio) was 0.2. Low levels of FFA in the samples indicate that little degradation of lipids had occurred during transport.

TABLE 1 Lipid class composition (mean ± S.D., expressed as relative percentage of total lipid content dry weight) in settlement stage bonefish (*Albula vulpes*) leptocephalus larvae collected at Long Caye, Lighthouse Reef Atoll, Belize ($n = 5$)

Lipid class	
HC	6.86 ± 1.94
WE-SE	4.72 ± 1.98
KET	14.05 ± 4.05
TAG	7.35 ± 3.04
FFA	7.36 ± 3.87
ST	35.38 ± 14.19
AMPL	9.02 ± 1.54
PL	15.25 ± 4.65
Total lipids (mg g^{-1} of dry weight)	33.75 ± 6.45

Note. HC, hydrocarbons; WE-SE, wax esters/steryl esters; KET, ketones; TAG, triacylglycerols; FFA, free fatty acids; ST, sterols; AMPL, acetone mobile polar lipids; PL, phospholipids.

TABLE 2 Neutral and polar fatty acid composition (mean ± S.D., expressed as percentage of total neutral and polar lipid dry weight) in settlement stage bonefish (*Albula vulpes*) leptocephalus larvae collected at Long Caye, Lighthouse Reef Atoll, Belize ($n = 5$)

	Neutral	Polar
C14:0	2.62 ± 0.993	2.18 ± 0.21
C16:0	19.91 ± 2.97	40.04 ± 5.14
C18:0	6.34 ± 0.81	12.71 ± 1.50
C20:0	0.64 ± 1.56	0.36 ± 0.02
C24:0	0.05 ± 2.29	0.34 ± 0.01
∑SFA	31.21 ± 6.79	57.84 ± 13.90
C15:1	0.78 ± 0.57	1.06 ± 0*
C16:1	3.84 ± 0.34	2.17 ± 0.10
C18:1	16.25 ± 6.23	10.42 ± 0.97
C20:1 n−9	1.05 ± 0.69	0.46 ± 0.11
∑MUFA	22.48 ± 6.27	14.47 ± 3.99
C18:2	3.03 ± 0.68	2.09 ± 0.27
C18:3 n−3	1.16 ± 0.26	0.65 ± 0.24
C18:3 n−6	0.80 ± 0.82	0.11 ± 0.05
C20:2	1.40 ± 0.08	0.35 ± 0.05
C20:4 n−6 (ARA)	4.25 ± 4.11	2.66 ± 1.56
C20:5 n−3 (EPA)	9.31 ± 2.69	3.59 ± 1.08
C22:5 n−6	1.82 ± 0.11	1.71 ± 0.36
C22:5 n−3 (DPA)	2.36 ± 0.26	1.64 ± 0.32
C22:6 n−3 (DHA)	24.98 ± 5.60	18.31 ± 2.35
∑PUFA	49.75 ± 7.25	31.87 ± 5.42
∑ n−3	36.75	24.18
∑ n−6	11.94	7.71
∑n−3: ∑n−6	3.08	3.13
	Total neutral (mg g^{-1})	Total polar (mg g^{-1})
	27.65 ± 1.09	13.39 ± 4.89

Note. The sums calculated above include fatty acids that were detected in this analysis but are not included in the table. Fatty acids not reported in this table include C15:0, C17:0, C21:0, C22:0, C14:1, C17:1, C22:1, C24:1, C20:3 and C22:4, whose relative percentages were each ≤3%. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

3.3 | Fatty acid composition and fatty acid ratios

Dividing the samples into neutral or polar lipids and characterizing the abundance of fatty acids within the pool can give more clues as to the diets of these larvae as well as defining the essential fatty acids required for cellular functions. There was a slight variation between the fatty acid composition of the neutral and polar lipid fractions. Total saturated fatty acids (SFA) were twice as high in the polar lipid fraction than in the neutral. The predominant FAs detected in the samples were C16:0

Heparin/heparan sulfate (HP/HS)		Chondroitin/dermatan sulfate (CS/DS)	
Total HP/HS[a] – 5 ± 1%	Percentage w/w	Total CS/DS[a] – 95 ± 1%	Percentage w/w
D0A0	23 ± 3.0	D0A0	12.33 ± 16.29
D0S0	7.33 ± 0.58	D0S0	34.00 ± 7.55
D0A6	5 ± 1.73	D0A6	45.00 ± 7.94
D2A0	1 ± 0[b]	D2A0	0**
D0S6	3.33 ± 1.53	D0S6	6.33 ± 1.15
D2S0	10.67 ± 1.53	D2S0	1.33 ± 0.58
D2A6	ND**	D2A6	1.00 ± 0[b]
D2S6	50 ± 3.46	D2S6	0.567 ± 0.24

TABLE 3 Heparin/heparan sulfate (HP/HS) and chondroitin/dermatan sulfate (CS/DS) content of settlement stage bonefish (*Albula vulpes*) leptocephalus larvae collected at Long Caye, Lighthouse Reef Atoll, Belize (percentage wet weight (w/w) of disaccharide classes expressed as mean of samples ± S.D.) ($n = 3$)

Note. Disaccharide nomenclature defined in Supporting Information Table S1. 0**, detectable amount that is less than 1%; ND**, not detected.
[a]Percentage of total isolate as calculated by adding together both detected glycosaminoglycan types (HP/HS and CS/DS only). Does not constitute the presence of hyaluronan or keratan sulfate.
[b]Standard deviation is 0 because disaccharide was only detected in one of the three samples.

and C18:1 n-9, as well as essential omega-3 and omega-6 FAs, eicosapentanoic acid (EPA), arachidonic acid (ARA) and docosahexaenoic (DHA) in both the neutral and polar lipid fractions (Table 2). Neutral lipids were dominated by polyunsaturated fatty acids (PUFAs) while polar lipids were dominated by SFAs and the polar lipid proportion contained nearly twice the amount of C16:0 as in the neutral portion (Table 2). Overall, total neutral lipids were higher than the total polar lipids. Fatty acid ratios were also calculated for all samples. In the neutral lipid fraction the DHA:EPA ratio was 2.68, whereas in the polar fraction the DHA:EPA ratio was 5.10. Another important ratio, C16:PUFA, was 0.40 and 1.26 in the neutral and polar lipids, respectively.

3.4 | Glycosaminoglycan content

GAG profiles were generated for two GAG groups, heparin/heparan sulfate (HP/HS) and chondroitin/dermatan sulfate (CS/DS). Overall, chondroitin/dermatan sulfate disaccharides were most abundant in the isolate obtained from the samples, accounting for 95% of the total GAG isolate. In the HP/HS fraction, D2S6 was the most abundant disaccharide (50%), whereas D2A0 was the least abundant (1%) (disaccharide nomenclature is defined in Supporting Information Table S1). In the CS/DS fraction, D0A6 was the most abundant disaccharide (45%) and D2S6 was the least (0.567%) (Table 3).

4 | DISCUSSION

Although prior studies have been conducted on the biochemical composition of other members of Elopomorpha, such as eels (Deibel *et al*., 2012) and other bonefish species (Mejri *et al*., 2018, 2021; Padrón *et al*., 1996), this is the first study of this kind focused on *A. vulpes*. This study therefore provides an initial definition of the biochemical composition of settlement-stage *A. vulpes* that can be used for comparison with wild-caught larvae from other regions and can serve as guide for understanding the dietary requirements of leptocephali reared in aquaculture settings. This study primarily focused on defining lipid characteristics because it is known that lipids are the main metabolic driver in leptocephalus larvae (Bishop *et al*., 2000; Glencross, 2009; Mejri *et al*., 2021; Tocher, 2003).

Recent efforts by our group led to inducing spawning in wild-caught, pre-spawning aggregation bonefish for the first time (Halstead *et al*., 2020; Mejri *et al*., 2019), as well as successful spawning of captive bonefish. However, the larvae from the spawning of wild fish and captive fish did not live past 10 days, indicating nutritional deficiencies and subsequent developmental difficulties (Wills, unpublished data). The information provided in this paper about the nutritional profile of wild-caught leptocephali will help to develop more targeted experimental treatments and husbandry methods for captive bonefish larvae.

4.1 | Lipids and lipid classes

The lipid class profiles of samples used for Iatroscan analysis were similar to those of eel leptocephali (Deibel *et al*. (2012). The dominant lipid classes identified here were sterols, phospholipids, ketones and acetone mobile polar lipids (AMPL), and the distribution of classes was similar to that found in the leptocephali from six different families of eel (Deibel *et al*., 2012). Overall, the eel leptocephali had the highest relative percentages of PLs but also exhibited high levels of TAGs, which were not detected in our samples. Across all samples of eel leptocephali, STs and PLs were highly conserved which is consistent with the high concentrations found in these leptocephali bonefish larvae. Additionally, the dominant lipid classes, PL, ST, AMPL, TAG, and FFA, accounted for 95% of total lipids in eel larvae, whereas the same classes accounted for about 80% of total lipids in bonefish larvae. Although the dominant lipid classes were similar, the relative percentages of each lipid class in eel leptocephali were different from those of bonefish. Eel leptocephali showed higher levels of phospholipids than bonefish, which may have been due to a difference in age and size. Lipid class profiles are known to change during the duration

of the larval stage and phospholipid content can change with age as larvae utilize them for cellular formation (Laurel *et al*., 2010). Lipid classes can be broken down into two groups: storage lipids and structural lipids. Phospholipids are important structural components within the lipid bilayer of cells and their abundance highlights their importance in early development for cellular formation (Glencross, 2009). Phospholipids are highly conserved across many species of marine fish larvae because of their importance to the integrity of the cell membrane (Rainuzzo *et al*., 1997). Storage lipids can be further divided into short-term and long-term energy storage lipids. Short-term energy storage lipids such as TAG are metabolized before long-term energy storage lipids such as WE-SE. The mean TAG:ST ratio (energy storage:cell structure ratio) was 0.2, which is close to that of other teleostean omnivores, suggesting a mixed diet (Carreón-Palau *et al*., 2018). Prior studies regarding early bonefish development have determined that wild bonefish oocyte lipid class and fatty acids have high levels of wax esters/steryl esters at the beginning of embryonic and early larvae development (Mejri *et al*., 2018, 2019, 2021). It is reasonable that WE-SE content is significantly lower in these late stage bonefish larval samples, as it is known that WE-SE reserves deplete over time during development due to its use in metabolic processes relating to growth (Glencross, 2009; Padrón *et al*., 1996). This is also supported by earlier findings by our group showing WE-SE as the main metabolite group during stages of development leading up to the settlement stage (Mejri *et al*., 2018, 2021).

4.2 | Fatty acid profiles

The neutral lipid portion was more abundant than the polar lipid portion across all samples. This is consistent with studies that determined fatty acid composition in eel leptocephali and in bonefish during earlier life stages (Deibel *et al*., 2012; Padrón *et al*., 1996). The essential fatty acids (EFAs) DHA, EPA and ARA are universally required by finfish for vital processes such as growth, muscular function (swimming) and neural development (Parrish, 1999; Sargent *et al*., 1999; Tocher, 2003). In a review study discussing the fatty acid requirements of marine fish larvae, DHA, EPA and ARA were found to be highly conserved during development across many species (Glencross, 2009; Izquierdo, 1996; Rainuzzo *et al*., 1997). The ratios of EFAs found in these samples were comparable not only to other elopomorph fishes (specifically eels), but also to other warm water marine finfish (Deibel *et al*., 2012; Izquierdo, 1996). Environmental factors and diet have some influence on EFA composition, but DHA is highly abundant in most species (Glencross, 2009). There is evidence of direct allocation of DHA for the development of neural and visual abilities (Tocher, 2003). Larvae diets are often enriched with these EFAs to promote growth and successful metamorphosis. In the neutral lipid fraction, PUFAs were the most abundant fatty acid class. DHA was the most abundant among the PUFAs, highlighting its importance as a dietary component. Total SFAs had the highest relative percentage in the polar lipid fraction, the most abundant of which was C16:0. SFAs are used alongside PUFAs for cellular membrane development. More specifically, SFAs are used to build the phospholipid bilayers of cells (Glencross, 2009). Because C16:0 is the main SFA retained in the polar fractions of the samples (greater than 40% of the total polar lipids), it is reasonable to assume that it is the main fatty acid metabolized for cellular membrane formation at this stage of development.

Another important fatty acid detected in all samples was 18:1, a monounsaturated fatty acid (MUFA), and 16:0, a saturated fatty acid (SFA), which were both found in high proportions in our samples. This is common in most teleost larvae and is observed across multiple species (Glencross, 2009). In particular, the C16:0 content in these samples is also comparable to the leptocephalus larvae of tropical eels and Californian bonefish (Deibel *et al*., 2012; Padrón *et al*., 1996). Other EFA ratios can indicate environmental conditions and diet, for example the DHA:EPA ratio can be used as a trophic biomarker that gives clues as to the diet of the fish. A DHA:EPA ratio >2 is indicative of good nutritional condition in marine heterotrophs (Padrón *et al*., 1996). With a ratio of 2.68 in the neutral fraction and 5.10 in the polar fraction, these samples are indicative of favourable environmental conditions in this location. These samples also contained high levels of the fatty acids 16:0, DHA, 18:0 and EPA, which is typical for organisms feeding at a low trophic level (Deibel *et al*., 2012). Flagellate and dinoflagellate microplankton are known to contain high amounts of DHA, making them a possible prey species for leptocephali. In captivity, larvae can be sampled and analysed to determine nutritional condition. This analysis can aid in the development of diets that help the larvae reach a similar nutritional profile as healthy wild-caught larvae. Finally, SFA, MUFA and PUFA totals in both the polar and neutral lipid fractions of our samples were also comparable to one of the few studies on the lipid composition of other bonefish species (Padrón *et al*., 1996). In particular, the high abundance of PUFAs in the neutral lipid fraction suggests a reliance on PUFAs as a substrate for metabolic processes. Although dietary fatty acids are a main source of energy for leptocephali, starvation can limit the availability of EFAs. This means that the larvae must depend on other stores of energy for basic functions such as swimming, while still allocating EFAs to development. In leptocephali, a secondary source of energy is stored within their gelatinous bodies in the form of complex polysaccharides called GAGs.

4.3 | Glycosaminoglycan profiles

Leptocephali possess a high concentration of GAGs as an additional energy store for developmental processes (Bishop *et al*., 2000). As GAGs are accumulated during metamorphosis, they are stored within the gelatinous matrix of the larvae and serve as a store of energy (adenosine triphosphate) that is dedicated to growth and development during early life stages (Bishop *et al*., 2000). These GAG stores allow the leptocephalus larvae to actively swim in the water column for long periods of time while also being resistant to starvation (Tsukamoto & Miller, 2021). In our study, the high relative proportion of chondroitin/dermatan sulfate indicates that this GAG group may serve an important metabolic role for bonefish during metamorphosis. In a prior study, the major GAG utilized by Albuliformes larvae was found to be keratan sulfate, with chondroitin sulfate following as a minor GAG (Pfeiler, 1998). Our study did not quantify keratan sulfate

or hyaluronan due to the lack of comparable methodology for isolation of these particular GAG groups and subsequent quantification with SAX-HPLC. In future studies, it would be ideal to quantify all GAG types to get a clearer picture of their purpose in developmental processes. The relative abundances of disaccharide classes in each sample can also give clues as to the diets of these leptocephali. It is postulated that these disaccharide building blocks are obtained from consumption of marine snow (Miller & Tsukamoto, 2020; Tsukamoto & Miller, 2021).

The findings from this study have implications for conservation efforts in the wild as well as in captivity and aquaculture settings. Our findings show an affinity in these larvae for EFAs such as DHA, EPA and ARA, which is consistent with studies on other species of elopomorphs and warm water marine fishes. Furthermore, our findings regarding lipid class composition are comparable to studies on bonefish and eel leptocephalus larvae, including the abundance of PUFAs in neutral lipids, suggesting that PUFAs are the main substrate for energy. This study corroborates past data about leptocephalus larvae and inspires new research directions in conservation and aquaculture. Increasing knowledge about this life stage in *A. vulpes* is helping to complete the full picture of the bonefish life cycle which, although the species is highly valued culturally and economically, remains somewhat of a mystery. Future studies can build on the findings above to develop new commercial aquaculture diets for warm water marine finfish, including elopomorph fishes like bonefish. These types of studies could also be enriched by larger macrocosm population studies that evaluate larval connectivity and habitat requirements at each life stage. As captive bonefish operations improve spawning strategies using this type of data and yield healthier larvae, we are steadily moving towards the goal of preserving the livelihood of this ancient species worldwide.

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AUTHOR CONTRIBUTIONS

V.U., S.M., J.M.S., A.J.A. and P.S.W. were all responsible for aspects of study design and manuscript writing, editing and reviewing. A.J.A., P.S.W. and J.M.S. were responsible for securing funding sources for this project. J.M.S. was responsible for collection and preservation of leptocephalus samples in Belize. V.U. and S.M. treated samples, conducted experimental procedures and performed calculations for analysis. Tables and figures were prepared by V.U. and S.M.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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SHORT NOTE

Novel deep-water spawning patterns of bonefish (*Albula vulpes*), a shallow water fish

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Abstract

Coastal marine fishes that form spawning aggregations most commonly exhibit a two-point movement pattern, with locations separated by migration: home range to spawning aggregation site and return to home range. However, the bonefish, *Albula vulpes*, partakes in a unique three-point spawning migration. Bonefish migrate up to 80 km from shallow water home flats to form nearshore pre-spawning aggregations (PSA) before moving offshore to spawn. Although these broad patterns have previously been documented, details of the offshore spawning-associated diving behavior have yet to be rigorously examined. Using active acoustic telemetry and sonar data in 2019 in Abaco, The Bahamas, we provide a complete account of bonefish offshore spawning movements and novel deep diving behavior to 137.9 m. Bonefish were continuously observed at depths ≥ 100 m for 2 h; a time period that included multiple depth changes and culminated in a spawning ascent to 67.3 m at 0.57 m s^{-1}. These new data on bonefish offshore movements and deep dives, coupled with CTD data, suggest that bonefish actively spawn at pycnoclines and thermoclines. Two previous tracking attempts (2013, 2018) at this location reflect spatiotemporal plasticity in spawning, a behavior counter to other aggregation forming fishes. This is the first detailed documentation of such deep spawning for a shallow water coastal fish species. The ecological motivation for diving to the deepest depths remains speculative. Future work must examine the dynamic relationship between bonefish diving behavior, spawning site selection, and oceanographic features.

Introduction

Spawning aggregations are a unique ephemeral reproductive strategy where fish come together en masse under certain environmental conditions to spawn. Spawning aggregations for coastal marine fishes may either be resident, where fish spawn within or near their home range, or transient, migrating beyond the bounds of a realized home range (Domeier and Colin 1997). Unlike the somewhat plastic selection of foraging grounds, and to some extent nursery habitat (Petitgas et al. 2012), transient spawning aggregations are highly predictable in that they are temporally and spatially restrictive, occurring at the same locations and times with regularity (Johannes 1978; Ciannelli et al. 2015). Spawning aggregation formation is synchronous with seasonal and physical cycles, allowing fish to migrate and arrive at spatially and temporally distant spawning grounds under consistent conditions.

Many identified spawning aggregation sites for coastal fishes have been located at promontories and slope drop-offs (Sadovy de Mitcheson et al. 2008), positioned to take advantage of hydrodynamic retention features (Johannes 1978). Migrations from a home range to a spawning aggregation often repeatedly follow landmarks and bathymetric features (Colin 1992; Mazeroll and Montgomery 1998; Feeley et al. 2018). For coastal aggregating fishes, the migration distance can be tens to thousands of kilometers to reach an aggregation site (Bolden 2000; Feeley et al. 2018). Some grouper species have been documented using unconsolidated proximal staging areas surrounding the aggregation site, where

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they exhibit no reproductive behaviors or coloration (Rhodes and Sadovy 2002a; Robinson et al. 2008). More closely associated to the spawning aggregation site, the courtship arena is where reproductive behaviors begin (Nemeth 2012). Delineation of the components of spawning habitat that surrounds an aggregation site can often be difficult (Feeley et al. 2018), as such the proximal areas have often been treated as a contiguous destination (Rhodes and Sadovy 2002b). Generally, migrations to and from spawning aggregation sites are uninterrupted (Nemeth 2012), following a home range—spawning aggregation—home range migration pattern (McCann et al. 2005). Bonefish (*Albula* spp.), which comprises 12 circumtropical shallow water species (Pickett et al. 2020), are a unique exception in that their migration is interrupted spatially and temporally by a distinct pre-spawning behavior (Danylchuk et al. 2019).

Albula vulpes (hereafter bonefish) are an economically and culturally important fish that supports recreational catch-and-release and artisanal fisheries throughout the Caribbean Sea and western North Atlantic Ocean (Adams et al. 2014). The estimated annual economic impact of the catch-and-release flats fishery, which includes bonefish, tarpon (*Megalops atlanticus*) and permit (*Trachinotus falcatus*), is US$50 million in Belize (Fedler 2014), US$169 million in The Bahamas (Fedler 2019), and US$465 million in the Florida Keys (Fedler 2013). Bonefish show high site fidelity to their home range, foraging for invertebrates over a habitat mosaic of intertidal sand flats, seagrass beds, mangroves, and hardbottom (Boucek et al. 2019; Brownscombe et al. 2017, 2019; Murchie et al. 2013). In The Bahamas, bonefish migrate from their shallow water home flats to nearshore deeper-water locations where they form pre-spawning aggregations (PSA). These activities occur over 4–7 days spanning the new and full moons of October through April (Danylchuk et al. 2011). The initial migration can cover distances of up to 80 km (Boucek et al. 2019) and culminates with an aggregation of 2000–5000 fish in 5–10 m of water (Danylchuk et al. 2011, 2019). At sunset bonefish begin "porpoising," where they gulp air at the surface (see Danylchuk et al. 2019), and then proceed to move offshore following the edge of the continental shelf (Danylchuk et al. 2011, 2019). Similar pre-spawning behavior and potential offshore migrations by *A. vulpes* have been observed in other locations such as Belize (Perez et al. 2019), Cuba (Posada et al. 2008), and Mexico (Zeng et al. 2019). Additionally, this behavior has also been observed in the congener *A. glossodonta*, in Kiribati (Johannes and Yeeting 2000) and Tetiaroa and Anaa, French Polynesia (Filous et al. 2019a, 2020). Spawning behavior of any kind has yet to be documented for the remaining ten albulid species.

Data describing the offshore spawning movements of bonefish are limited, but of high importance due to the economic value and conservation needs of the species and genus throughout their distributions (Adams et al. 2014; Fedler 2013, 2014, 2019; Filous et al. 2019b). Danylchuk et al. (2011) used both passive and active acoustic telemetry techniques to characterize the timing and location of offshore spawning movements in Cape Eleuthera, The Bahamas; the first study to do so. Logistical and technological limitations of both the passive and active acoustic equipment precluded any determination of spawning depth, but did reveal consistent offshore movement behaviors towards the shelf edge and waters exceeding 42 m, followed by a return to the shallow water flats habitat. Danylchuk et al. (2019) provided a more detailed description of pre-spawning behavior and offshore movements from active acoustic tracking efforts from 2013 and 2014 in Andros and Abaco, The Bahamas. The 2013 Abaco active tracking event yielded the first ever recording of diving movements during the offshore spawning migration. However, the description of fish vertical movement through the water column was restricted due to the depth rating (50 m) of the pressure sensor within the acoustic tags. In this study, we build upon the efforts and data of Danylchuk et al. (2019), presenting the spatiotemporal variability of spawning movements in Abaco. Most significantly, we document for the first time the complete deep diving and presumed spawning behavior of bonefish via several additional active tracks.

Materials and methods

Study site

The study took place along the southern shore of Abaco, The Bahamas. Previous work by the authors identified a bonefish PSA at Long Bay that occurs with regularity that allows for repeatable observation. Long Bay is a south-facing bay along a western-extending point, comprised of a mosaic of marl, seagrass (*Thalassia testudinum* and *Halodule wrightii*) and patch reef habitat. To the south of Long Bay the continental shelf narrows, and at 1.5 km offshore the benthos transitions to a steep vertical drop-off of more than 1000 m. The location of Long Bay provides 180-degree protection from NNW to SSE winds and access to the adjacent north bay with protection from S winds. Ocean surface currents typically flow SSE to NNW with quasi-permanent cyclonic-gyres forming on the west-side of Abaco and south of Grand Bahama (Chérubin 2014).

Fish collection and tag deployment

Offshore spawning migrations from the PSA site were actively tracked with acoustic telemetry in 2013 (see Danylchuk et al. 2019), 2018, and 2019. Active tracking methods were chosen rather than the deployment of a

passive acoustic telemetry array due to the logistical and technological constraints of working in offshore waters of > 1000 m, and experience gained by previous efforts detailed in Danylchuk et al. (2011, 2019). Methods for fish collection were maintained throughout the study, while tag selection and deployment methods changed across iterations of the study as more information about bonefish diving behavior was revealed.

Upon locating the PSA and visually confirming behaviors indicative of an imminent offshore migration (see Adams et al. 2019 for PSA identification method), fish were captured using hook-and-line and cast net (0.355 mm monofilament, 2.4 m radius × 31.8 mm stretch mesh). Sex was determined by ventral palpation or cannulation to identify females. We tagged bonefish at a 1:1 sex ratio. Implantation of acoustic telemetry tags was conducted in a small plastic cooler (33 L) filled with ambient seawater with the fish held ventral side up. Two tagging methods were used, with results yielded from the 2013 tags and tagging method informing future tag choice and tagging method. In 2013, bonefish were gastrically tagged with Vemco continuous acoustic transmitters with pressure sensors (V9P-2H, 9 mm diameter, 21 mm in length, 1.6 g in air, 2000 ms transmission period). These tags had a pressure sensor depth limit of 50 m (see Danylchuk et al. 2019). In 2018 and 2019, Vemco continuous pressure and temperature tags (V9TP, 9 mm diameter, 31 mm long, 4.9 g in air, period 1000 ms) with a 250 m depth limit were surgically implanted (Innovasea Systems Inc., Massachusetts). Each tag emitted a unique frequency at an interval of 1000 ms with an assumed detection range of 300 m (Melnychuk and Christensen 2009). The maximum number of at large V9TP continuous acoustic tags was limited to 6 due to the number of available unique frequencies manufactured. A 2 cm incision was made on the fish's ventral side, just posterior of the pelvic fins, with the transmitter inserted into the peritoneal cavity. The incision site was closed with two interrupted sutures (Ethicon 3–0 PDS II; Johnson and Johnson, New Jersey). Bonefish were allowed to recover in an aerated cooler to ensure that stress levels were reduced and equilibrium was constantly maintained prior to release to maximize survival (Brownscombe et al. 2013). Once recovered, bonefish were then placed back into the PSA to further reduce the likelihood of predation (see Adams et al. 2019).

The number of individuals tracked offshore from the PSA location were less than the number of tags deployed due to predation events, tag failures, and lost contact with tagged fish during tracking efforts. Initial offshore movements of the entire PSA school were visually confirmed, though the number of individuals successfully tracked through the initial dive ranged from one (2013 and 2019) to three (2018) individuals.

Data collection and analysis

Fish were actively tracked using a Vemco VH-110 directional hydrophone connected to a Vemco VR100 receiver and deck box (InnovaSea Systems Inc., Massachusetts). Based on challenges in previous tracking attempts, in 2019, we modified the directional hydrophone mount to create a vertically (i.e., pitch) modulated directional hydrophone, allowing the directional hydrophone transducer head to be articulated vertically from the standard horizontal position. Offshore school movements were also monitored using hull-mounted Compressed High-Intensity Radiated Pulse (CHIRP) and sidescan sonars (Axiom 600 W sonar and RV-212 transducers; Raymarine, United Kingdom). GPS position, water temperature (2018 and 2019 only), depth, and time were recorded every 3 s. In 2019, water property data were collected daily along transects from shore to the edge of the continental shelf using a CastAway CTD rated to 100 m (SonTek YSI, Xylem Inc., New York). Additional CTD casts were conducted during active tracking and at the culmination of the spawning event. Data visualization and estimations of rates of movement were constructed in R 3.4.1 (R Core Team, Vienna, Austria) and tracking maps were constructed using ArcMap 10.3.1 (ESRI, Redlands, CA).

Total migration path distance was estimated due to the periodically circuitous path taken by the boat while following the aggregation within the 300 m detection range. Therefore, horizontal movements were described as straight-line distances with coarse directional changes, and rates of movement were estimated over time periods of straight-line surface movement. Dive and ascent movement rates were calculated using simple linear regression, and the upper quartile ($Q3$) of the instantaneous rates of movement reported as a measure of the maximum. The upper quartile was selected as a representation of maximum movement rates to filter the inherent noise in acoustic telemetry data, which increases with range (Melnychuk and Christensen 2009).

Results

Tagging

In 2013, Danylchuk et al. (2019) tagged two females (430 ± 28 mm FL) and three males (409 ± 42 mm FL) with Vemco V9P-2H tags. In 2018, three females (436 ± 46 mm), two males (421 ± 5 mm), and one unknown (405 mm) were tagged with Vemco V9TP tags. In 2019, two females (520 ± 5 mm) and two males (450 ± 0 mm) were tagged with Vemco V9TP tags. Not all tagged fish were successfully tracked, as some fish emigrated, were lost to predation, or experienced tag failure. The resulting

number of fish tracked across 2013, 2018, and 2019 were 1, 3, and 1, respectively. We include the Danylchuk et al. (2019) data here to enable description of the spatiotemporal variability in spawning-related movements.

On 16 November 2013 at 12:23 EST, one night before the full moon, all four tags were deployed. However, signals from three of the four tagged bonefish were lost shortly thereafter due to predation or emigration. On 18 November 2018, five nights before the full moon, from 13:22 EST to 13:32 EST three of the six total fish were tagged. The signal was lost for two fish at approximately 15:30 EST and 15:34 EST. The PSA did not make any offshore movements by 21:31 EST, and tracking efforts were terminated. The following morning no tags were detected near the PSA location. On 19 November 2018, four nights before the full moon, at approximately 17:11 EST the final three of six total fish were tagged. On 8 November 2019, four nights before the full moon, two of the four total fish were tagged. The PSA remained within Long Bay for the rest of the evening and two more days. One additional fish was tagged each day, of which the last of the four total tagged fish was lost to a predation event approximately 1 h post-release. The three fish that were at large from the 8th to 10th remained within close proximity to the PSA location, occasionally moving in an offshore direction each night, but stopping prior to leaving the reef, only to return back to the PSA location. Two of the three fish were lost shortly after the fish began moving offshore, leaving the first fish tagged (Female, 525 mm) on 8 November 2019 to be tracked.

Lateral movement

PSA formation and movement offshore varied in relation to lunar and solar position. In 2013, the PSA formed one night before the full moon and the aggregation of bonefish proceeded to move offshore that night at 17:06 EST, 2 min before sunset. In 2018, the PSA formed five nights before the full moon and didn't move away from the PSA location until four nights before the full moon at 17:11 EST, at sunset. In 2019, the PSA formed four nights before the full moon and did not commit to moving offshore until one night before the full moon at 18:15 EST, 55 min after sunset.

Generally, fish followed the steep drop-off of the continental shelf as they migrated southward and up-current (Fig. 1), moving at depths of 0–5 m. Maximum horizontal movement rates were calculated as 1.21 m s^{-1} in both 2013 and 2019, and 0.62 m s^{-1} in 2018. On all tracks, porpoising behaviors sporadically occurred during offshore movement, lasting 5–10 min. Porpoising was noted four times throughout the 2019 spawning migration. The number of times porpoising occurred during offshore migration was not noted in 2013 and 2018. The distance traveled, location of, and timing of the initial dive varied considerably across all years (Figs. 1, 2). The single fish tracked in 2013 moved the shortest distance, traveling 0.9 km (±0.3 km) at 143° SE

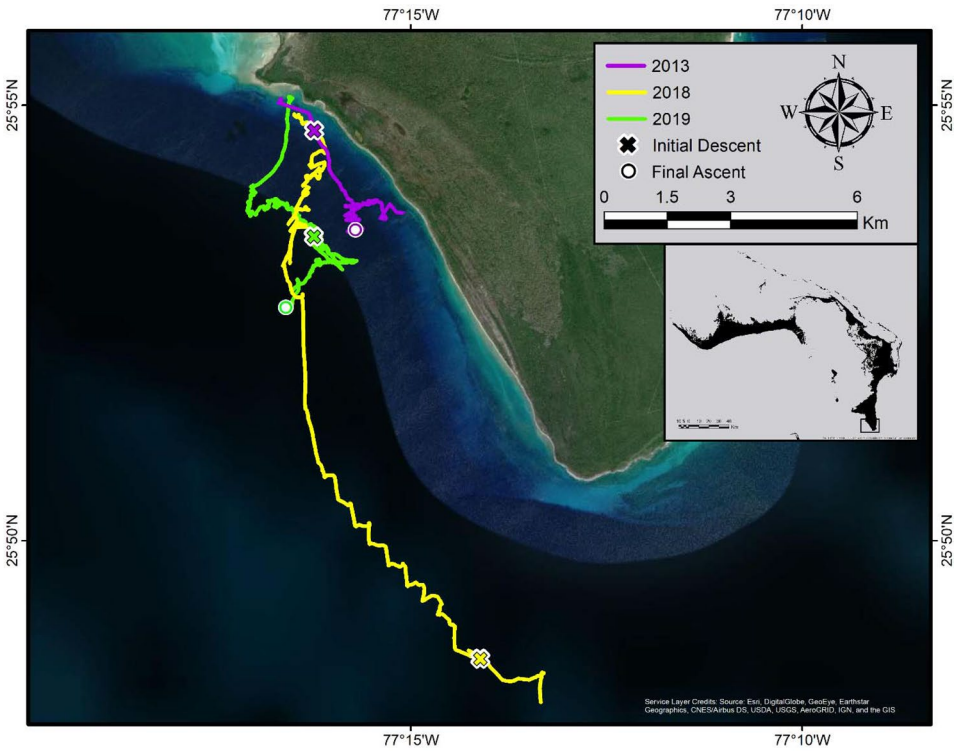

Fig. 1 Spawning migration pathways of bonefish tagged at a PSA location in South Abaco, The Bahamas. Across all 3 years, an initial descent from surface movements was observed (indicated by X). In 2013 and 2019, the final ascent was observed (indicated by O)

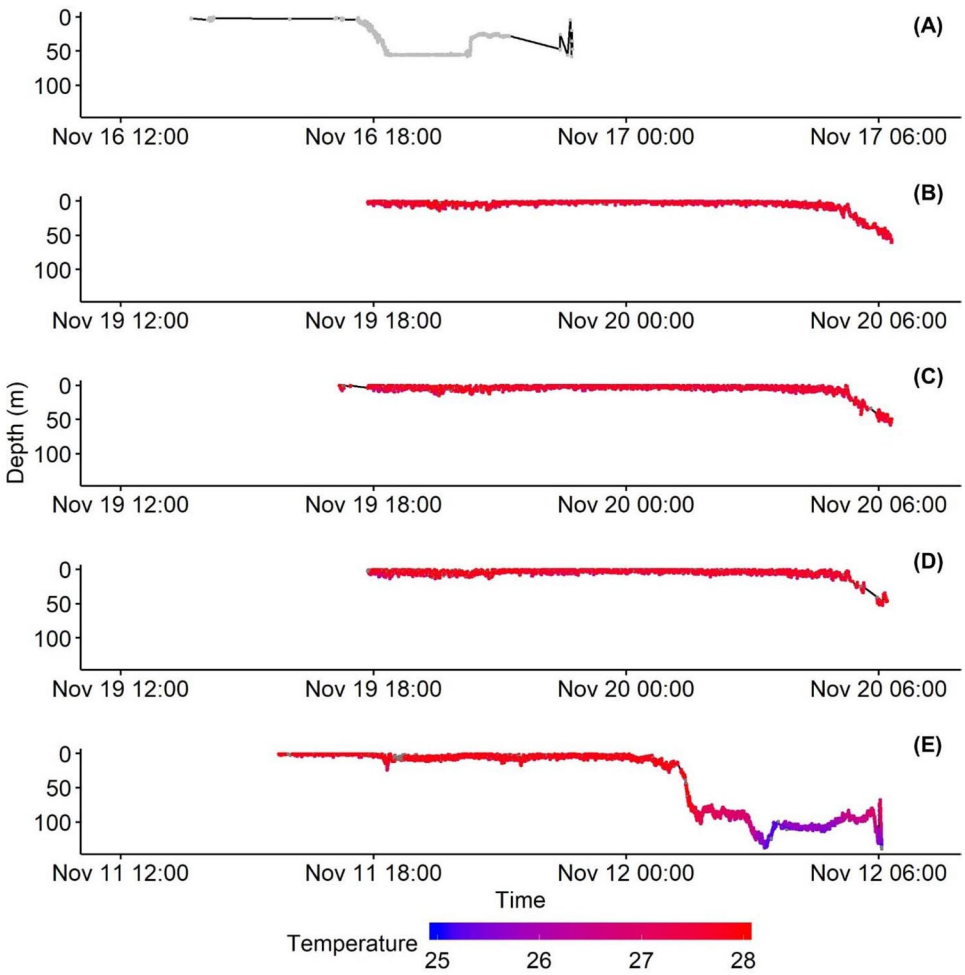

Fig. 2 Dive profiles for actively tracked bonefish in 2013 (**a**) (Danylchuk et al. 2019), 2018 (**b**–**d**), and 2019 (**e**). Acoustic telemetry tags were depth limited to 50 m in 2013, and tags did not have a temperature sensor. Active tracking in 2018 ended when the fish were lost descending below 58 m. Bonefish were tracked in 2019 up until the tag was ejected in a likely spawning event

from the PSA site to the initial descent site, arriving 38 min later at 17:38 EST. The fish continued their lateral movement at depth, moving another 2.6 km (±0.3 km) over 159 min before ascending and moving toward shore. The track was ended at this time because previous studies using passive acoustic telemetry (Danylchuk et al. 2011) and local ecological knowledge had already described the return movement.

In 2018, the aggregation moved the furthest distance, with the three tagged bonefish migrating 17.3 km (±0.3 km) from the PSA to the descent site beyond the southern tip of Abaco. The migration to the descent site took 327 min from when the fish began moving offshore, arriving at approximately 05:15 EST. The fish made an initial offshore movement with a heading of 199° SSW, followed by a prolonged migration 153° SSE. The fish continued to move SSE for 1.7 km (±0.3 km) before the tag signals were lost in rough seas.

In 2019, the fish moved 5.1 km (±0.3 km) from the PSA to the descent site, arriving 257 min later at 00:32 EST. Before the initial dive, the aggregation began moving 191° S before abruptly turning to 127° ESE. The fish continued an ESE heading while at depth before heading 236° SW further beyond the drop-off, covering another 3.1 km (±0.3 km) before completing a likely spawn.

Vertical movement

Depth profiles in 2013 (see Danylchuk et al. 2019) and 2018 provided limited information due to equipment limitations and rough seas. However, in 2019, a complete likely spawning cycle was observed (Fig. 3). The first dive to 25.3 m began on 12 November 2019 at 00:32 EST, 466 min after leaving the PSA site. After its initial descent, the bonefish underwent six descents and seven ascents (Table 1) with the collective aggregation of bonefish being observed along the way by sonar (inset Fig. 3). The second descent was the longest continuous dive with the greatest difference in depth, from 13.2 to 102.1 m over 33 min (0.04 m s^{-1}; $Q3 = 0.57$ m s^{-1}). The dive is composed of two measurably distinct rates of descent, with the first portion from 13.2 to 69.1 m descending at 0.07 m s^{-1} and the second portion from 69.1 to 102.1 m descending at 0.02 m s^{-1}. During the dive, hydrostatic pressure increased + 871.8 kPa to 1001.2 kPa. The deepest

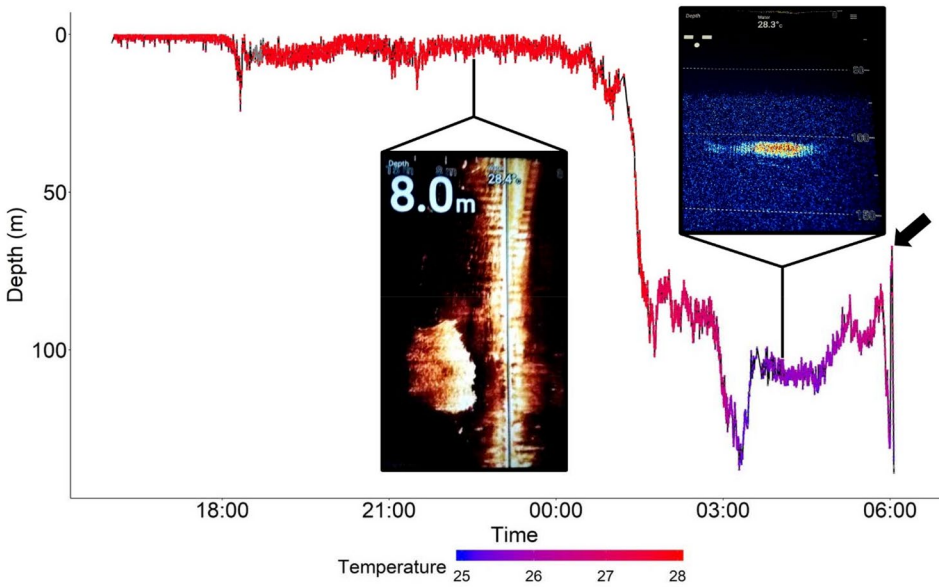

Fig. 3 Dive profile of the female bonefish tracked during the 2019 spawning migration, colored to temperature transmitted by the acoustic telemetry tag. Sidescan and CHIRP sonar images of the aggregation are inset at times that the boat passed over the moving aggregation. Black arrow indicates tag ejection

Table 1 Summary of 2019 bonefish offshore movements

Movement	Depth start (m)	Depth end (m)	Abs. depth change (m)	Time elapsed (min)	Overall rate (m s^{-1})	$Q3$ instantaneous rate (m s^{-1})
Descend	3.1	25.3	22.1	29	0.01	0.40
Ascend	25.3	11.6	13.7	4	0.03	0.57
Descend[1]	13.2	69.1	55.9	15	0.07	0.54
Descend[1]	69.1	102.1	33.0	18	0.02	0.57
Ascend	102.1	78.2	13.9	5	0.07	0.55
Descend	74.8	94.2	19.4	5	0.06	0.57
Ascend	92.4	78.1	13.3	3	0.04	0.40
Descend	78.1	97.6	19.5	10	0.02	0.60
Descend	85.6	137.9	51.1	30	0.02	0.57
Ascend[2]	137.9	120.3	17.6	6	0.03	0.40
Ascend[2]	124.8	104.4	20.4	4	0.08	0.56
Ascend	112.3	87.3	25	34	0.01	0.53
Ascend	98.6	82.7	15.9	12	0.02	0.40
Descend[3]	82.7	114.5	31.8	4	0.12	0.45
Descend[3]	106.6	131.0	24.4	2	0.14	0.55
Ascend	131.0	67.3	63.7	2	0.57	1.15

Movements are listed in chronological order, with incongruencies in subsequent start and end depths temporally separated by horizontal and vertical oscillatory movements. In instances where single movements are composed of distinct movement rates, movements are detailed by their components and denoted by the same superscript. Times are rounded to the nearest whole minute

dive started at 02:47 EST from 85.6 m and ended at a depth of 137.9 m (1352.3 kPa). The final descent began 4.5 km (±0.6 km) from the PSA site, at 05:52 EST. Fish descended to 129.9 m in two movements interrupted by a 1 min ascent. Both movement rates exceeded 0.12 m s^{-1} ($Q3 = 0.45$ m s^{-1}; $Q3 = 0.55$ m s^{-1}). Depth was maintained near 129.9 m for 1 min before the final measured ascent at approximately 06:00 EST. The final ascent plateaued abruptly at 74.4 m, oscillating for 1 min until reaching a depth of 67.3 m, upon which the tag was ejected from the coelomic cavity and proceeded to fall at a constant rate. The ascent was completed in 2 min, with the fish moving at a rate of 0.57 m s^{-1} ($Q3 = 1.15$ m s^{-1}, max = 2.55 m s^{-1}). During the final ascent, fish experienced a 613.9 kPa decrease in pressure over 2 min, and a 962.3 kPa decrease in pressure from the deepest dive. The entire track lasted

14 h and 16 min, from departing the PSA site to presumed spawning, with fish occupying depths > 100 m for approximately 2 h.

CTD profiles

CTD casts were taken each of the 3 days prior to the 2019 spawning run and during the spawning track. These revealed an offshore pycnocline and thermocline, formations that were not present in waters < 2.2 km from shore and inside the 200 m contour. Three days prior to the spawning run, at a location 3.1 km SE of the location where the spawning ascent occurred, the initial signal of a pycnocline was detected at 68.4 m (1024.0 kg m^{-3}) with a thermocline at 67.5 m (27.7 °C). The CTD cast at the location of the final spawning ascent displayed pycnocline and thermocline signals at 75.9 m with measurements of 1024.0 kg m^{-3} and 27.7 °C, respectively (Fig. 4).

Discussion

Through 3 years of effort we were able to iteratively build upon knowledge of bonefish offshore movements and diving behavior associated with presumed spawning off South Abaco. PSAs in South Abaco formed 1–5 days prior to the full moon, with offshore movements beginning from 2 min before to 55 min after sunset. Active tracking revealed that bonefish show substantial spatial and temporal variation in spawning locations, counter to high site fidelity in forming PSAs (Johannes and Yeeting 2000; Danylchuk et al. 2019; Filous et al. 2020). Offshore movements were directed toward the nearest southern point, as seen in Cape Eleuthera (Danylchuk et al. 2011). However, the departure angles and migration paths taken were different each of the 3 years with the location and time where the fish began to descend varying considerably. The maximum rate of horizontal movement also appears to reflect inversely on the travel distance to where bonefish begin to descend to depth. In 2019, the first detailed dive profile logged bonefish moving offshore and diving to depths > 137 m during their spawning migration, with spawning likely occurring at 67.3 m, following a rapid ascent. The spawning aggregation spent > 2 h below 100 m, as indicated by the acoustic telemetry tagged fish and corroborated by CHIRP sonar images. Tracking data and CTD data also suggest that bonefish spawn at depths associated with pycnocline and thermocline features. These data are the first detailed documentation of the novel deep diving movements of spawning bonefish, and the first documentation of such spawning depths for shallow water coastal species.

The spawning migration of bonefish beyond the continental shelf break and diving to such great depths, as documented in this study, are novel behaviors within the coastal marine teleost assemblage, and are certainly unique for bonefish, which spend a majority of their time in waters < 1 m deep (Danylchuk et al. 2011; Murchie et al. 2013; Boucek et al. 2019). Generally, coastal marine fish that form transient spawning aggregations migrate uninterrupted between their home range and spawning aggregation site (Nemeth 2012), which form with high spatial and temporal fidelity (Sadovy de Mitcheson et al. 2008). Bonefish

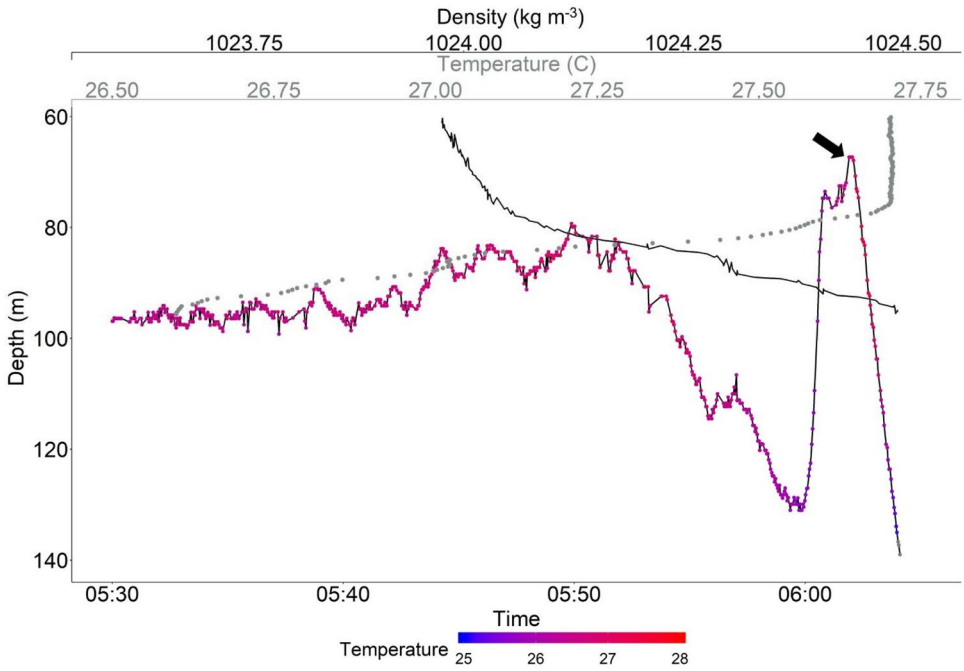

Fig. 4 Final 2 h of the 2019 dive profile, as portrayed in Fig. 3. Tag ejection at the pycnocline and thermocline can be seen from 06:02 EST. Colored line depicts ambient temperature of bonefish at depth, black line is CTD density, gray dots are CTD temperature, black arrow indicates tag ejection

reproductive behavior differs from other species that share adjacent habitats (coral reefs) in that they show high fidelity to a pre-spawning location and apparent variability in actual spawning location. Migration to reef promontories is a common strategy for many coastal marine fish species (Johannes 1978; Kobara and Heyman 2006). By moving further offshore and away from the reef, fish are able to take advantage of lower zooplanktivore densities, and retention features such as slow-moving coastal boundary layers (Nickols et al. 2012) and gyres (Johannes 1978; Lee et al. 1994; Lindeman et al. 2001; Paris and Cowen 2004). Bonefish may spawn in habitat independent of bathymetric features, such as promontories, and instead cue in on the formation of frontal systems.

The CTD cast taken immediately after the acoustic telemetry tag was ejected from the fish showed signals of the upper stratification of a pycnocline and thermocline at 75.9 m. This was 7 m below the depth that the tagged bonefish presumably spawned, but the clines are exactly at the depth where "mixing" movements, balling and swirling behaviors when spawning (Heyman et al. 2005), can be observed in the dive profile (Fig. 4). The respiratory and reproductive physiology of bonefish lends support for the expulsion of the acoustic telemetry tag (which measured 9 mm diameter, 31 mm long) from the peritoneal cavity during a spawning rush as the most parsimonious explanation. Bonefish are gymnovarian spawners; as such, their eggs are released from the ovary into the coelomic cavity before being expelled via the oviduct. It has been hypothesized that the porpoising pre-spawn behavior sequesters air into the swim bladder at the surface, and that the diving and spawning rush motion acts as a pneumatic assist to expel eggs (Danylchuk et al. 2019). However, the depth and temperature data acquired in this study suggest that the surface volume of the swim bladder is not enough. A 172 cc swim bladder (Danylchuk et al. 2019) would compress to 1.2 cc at 137.9 m and only expand to 2.4 cc at 67.3 m. In order to create sufficient gas expansion for a pneumatic assist to occur, gas must be sequestered at depth. We hypothesize that this is accomplished using swim bladder gas gland cells located throughout the epithelium of the swim bladder, a trait that may be conserved within the superorder Elopomorpha and identified in the also physostomous European eel, *Anguilla anguilla* (Prem and Pelster 2000; Smith and Croll 2011). Increases in swim bladder volume at depth are further supported by the target strength of the sonar backscatter from the aggregation at depths > 100 m (inset Fig. 3.), which would be decreased or absent in a physostomous fish with a compressed swim bladder following Boyle's Law (Blaxter et al. 1979; Mukai and Iida 1996; Mukai and Foote 1997; Benoit-Bird et al. 2003).

It is possible that the tagged bonefish may have been consumed by a predator along the offshore migration or that the tag ejection may have been caused by a predator consuming the tagged fish, as sharks were observed via sidescan sonar following the aggregation offshore at depths < 8 m (inset Fig. 3). However, the data and observations suggest no predation event occurred. The rate of movement preceding tag ejection was consistent with movement rates throughout the offshore migration, and sonar images of the spawning migration confirmed we were tracking the bonefish aggregation and not a predator (inset Fig. 3). Additionally, no excessive rates of movement that may be associated with predator avoidance were observed, and previous observations of sharks consuming tagged bonefish in shallow waters resulted in the shark leaving the aggregation (pers. obs.).

Oscillations above and below the pycnocline were evident throughout the 5 h that the fish spent at depth in 2019. These oscillations may have been an exploratory behavior, where bonefish were sampling water column conditions (i.e., pycnocline and thermocline) before identifying where to spawn. This oscillatory behavior has not been examined for any fish species in relation to oceanographic features, but water column position of larvae is often correlated with stratified oceanographic features (Moser and Smith 1993; Sabatés and Olivar 1996; Sánchez-Velasco et al. 2007). This correlation has been documented in multiple Elopomorphs, with anguilliform larvae having been found at 50–100 m depth (Tsukamoto 1992) and larvae of *Albula* sp. being found as deep as 200 m but in the highest abundance when associated with a stable stratified layer outside of a cyclonic eddy in the Gulf of California (Sánchez-Velasco et al. 2013). These studies support the hypothesis that other Elopomorphs actively search out similar conditions. Therefore, pelagic spawning habitat for some fish, such as bonefish, may be subject to inspection and selected for based on perception of physical characteristics of the water column. This behavior would explain why the tag was not spawned out of the female fish until the final upward rush, aided perhaps by the change in hydrostatic pressure (Domeier and Colin 1997; Graham and Castellanos 2005; Whaylen et al. 2004), which likely facilitated gamete release and acoustic tag expulsion into the water column; a fate not observable in 2013 due to the gastrically implanted tags. Additionally, it is possible that multiple spawning rushes occurred with different individuals spawning during each event, with the tagged fish not spawning or completing its spawn until the seventh ascent. Measurements of movement rates throughout the spawning run indicate that the final descent and ascent were unique, reaching rates of movement at least two and seven times greater than other measured movement rates, respectively. As such, it is likely that the tagged bonefish only spawned once over the observation period. However, it remains unknown whether bonefish release gametes multiple times throughout a spawning run.

There are many benefits to spawning within a frontal system boundary layer, such as a pycnocline or thermocline. Pycnocline boundary layers can act as a retention feature for planktonic organisms, which includes larval fish and prey that support early growth (Kiørboe et al. 1988; Bjorkstedt et al. 2002; McManus and Woodson 2012). Retention within the boundary layer provides organisms an environment independent of the larger masses of water, subjecting them to reduced turbulence and flow velocities (Doostmohammadi et al. 2012), and likely a more predictable and stable dispersal mechanism (Paris and Cowen 2004; Nickols et al. 2012). While we know these frontal systems play an important role in the transport of bonefish larvae (Zeng et al. 2019) during their 41–71 days pelagic larval duration (Mojica et al. 1995), dispersal pathways from the Providence Channel at depths > 0.5 m have not yet been quantified. These data could provide crucial information on which bonefish populations of The Bahamas are being supported by the spawning aggregations observed herein off South Abaco.

These studies in The Bahamas can provide the foundation for a conceptual model in studying bonefish spawning movements, which can then be applied to other populations of *A. vulpes* and potentially other albulid species. Further observations of bonefish diving behavior should be made throughout the Caribbean. Locations with gradually sloping coastal bathymetry, like the Florida Keys, and also nearshore drop-offs like South Abaco and Cape Eleuthera will provide additional perspective on bonefish spawning habitat selection. Observations of other elopomorph spawning events, beyond the presence of pelagic larvae, are needed to confirm that the diving behavior exhibited by bonefish are not unique to the species. We still do not understand the physiology and ecology of why bonefish dive to depths > 137 m. Adams et al. (2019) hypothesized that egg hydration is occurring as bonefish descend, while Danylchuk et al. (2019) hypothesized the behavior is related to pneumatic assist for releasing eggs, and here we propose a modified pneumatic assist hypothesis accounting for the deep diving depths and spawning movements. Additional work examining the significance of diving to the deepest depth, and the physiological mechanisms that allow bonefish to compensate for repeated, rapid and extreme pressure changes should be pursued.

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Author contributions SL, AA, AD, CL, and MA were all involved in field operations and data acquisition, with AA and AD conducting work in 2013, and SL, AA, CL, and MA conducting work in 2018 and 2019. AA performed surgery and tagging procedures. The text, analyses, table, and Figs were prepared by SL, and internally edited and reviewed by AA, AD, CL, and MA.

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Code availability The R code written and executed within R Studio may be provided through a request to the authors. No novel data handling schemas or tools were created in the analysis process of this research.

Compliance with ethical standards

Conflict of interest The submitted work was not carried out in the presence of any personal, professional, or financial relationships.

Ethical approval The non-profit institution Bonefish and Tarpon Trust conducted the tagging, and does not have a formal animal care and safety requirement for research. All precautions were taken to ensure fish survival, and BTT uses commonly accepted and applied methods for fish care. All measures were taken to minimize animal pain and suffering during collection and tagging. Research methods and procedures were reviewed and approved by The Bahamas Ministry of Agriculture and Marine Resources under permit: MA&MR/FIS/17.

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Behavioral observations of bonefish (*Albula vulpes*) during prespawning aggregations in the Bahamas: clues to identifying spawning sites that can drive broader conservation efforts

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Abstract Bonefish are typically thought of as 'flats fish' that reside in shallow, tropical and subtropical nearshore coastal waters. However, evidence from tagging and acoustic tracking studies indicate that bonefish migrate to staging areas, form large aggregations (>5000 individuals), and then move to deep-water drop-offs at dusk to spawn. Because the offshore spawning occurs under the cover of darkness, visual observations of the actual bonefish spawning events are not possible. Fortunately, behaviors during prespawning aggregations provide clues related to putative spawning bouts. For multiple locations in The Bahamas, we report on repeatable, predictable behaviors of bonefish in large prespawning aggregations. Just prior to moving from shallow to deeper waters, bonefish are observed breaking the water surface ('porpoising') and then returning to the aggregation. Bubbles are then observed emerging from the swirling aggregation; potentially gas being released from the bonefish. Ventral nudging, when one fish rubs its snout on the ventral region of another, has also been observed, but not as consistently as porpoising. Based on the depth profile of acoustically tagged fish, we hypothesize that porpoising is related to 'pneumatic assist' for egg release during spawning bouts. Although these observations provide circumstantial evidence that these behaviors are related to spawning, they are consistent with the prespawning behaviors of other marine fish that broadcast spawn. More research is needed to actually document a spawning bout; however, these clues in addition to other traits may be enough to encourage conservation measures to protect this important life history event for bonefish.

Keywords Spawning aggregation · Bonefish · Behavior · Porpoising · Offshore movement

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Introduction

Spawning is a critical life history event that ensures the maintenance of fish populations (Stearns 1978). For many marine fishes, particularly those that broadcast spawn, this event is facilitated through the formation of large aggregations (Sadovy de Mitcheson et al. 2008).

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Aggregating behavior associated with spawning brings fish together that normally compete for other resources during the rest of their lives, and has evolved to occur at locations that increase the likelihood that successfully fertilized eggs will survive and disperse to areas where post-settlement larvae can be recruited to the population (Dahlgren et al. 2008).

Determining that a fish aggregation is associated with spawning can be defined by characteristics and behaviors that are not common to the fishes' everyday lives (Domeier and Colin 1997). Domeier (2012) provides specific criteria for validating that a fish aggregation is related to spawning, including the gathering of reproductively active conspecifics at densities and/or numbers higher than those found in the area during non-reproductive periods, as well as courtship behaviors not normally observed when not in aggregations. These traits have been used to document spawning aggregations for coral reef fishes, such as Nassau grouper (*Epinephelus striatus*, Bolden 2000; Whaylen et al. 2004) and red hind (*Epinephelus guttatus*, Sadovy et al. 1994; Beats and Friedlander 1998), and coastal species such as common snook (*Centropomus undecimalis*, Lowerre-Barbieri et al. 2003) and two species of bonefish (Johannes and Yeeting 2001, *Albula glossodonta*; Danylchuk et al. 2011, Adams et al. 2018, *Albula vulpes*).

Bonefish (*Albula* spp.) are a group of 12 species of benthivorous fish that are typically associated with shallow tropical and sub-tropical flats habitats (Alexander 1961; Ault 2008; Murchie et al. 2013). However, using passive and active tracking with acoustic telemetry, Danylchuk et al. (2011) was first to demonstrate that *A. vulpes* in Eleuthera, The Bahamas, seasonally moved away from their flats, aggregated at 'transitional' habitats near a deep-water drop-off, and then moved offshore. These aggregations coincided with the new and full moons between December and April, and movements offshore happened at dusk, independent of tidal cycle (Danylchuk et al. 2011). These movement patterns documented by Danylchuk et al. (2011) fit well with the criteria outlined by Domeier and Colin (1997), and were later used by Adams et al. (2018) and Boucek et al. (2018) to locate other potential bonefish spawning aggregation sites in The Bahamas.

Given that bonefish are prized by recreational anglers (Danylchuk et al. 2008) and support local and regional economies in developed (Florida Keys; Fedler 2013) and developing nations (e.g., Bahamas, Fedler 2010; Belize, Fedler 2014), mapping the specific locations of spawning aggregation sites could be critical for the use of appropriate management tools to ensure their protection (Coleman et al. 1996; Domeier and Colin 1997; Roberts and Hawkins 1999; Musick et al. 2000; Sala et al. 2001; Adams et al. 2018). One caveat is that many government agencies likely do not have the financial and logistical capacity to undertake detailed tracking studies that initially allowed Danylchuk et al. (2011) and Adams et al. (2018) to locate bonefish prespawning aggregations. As such, identifying highly conspicuous behaviors that are solely related to bonefish prespawning aggregations and spawning activity could prove essential for identifying the location of spawning sites, especially across vast geographic areas.

During the study by Danylchuk et al. (2011) several behaviors were observed that were not previously documented for *A. vulpes* (or any other species of bonefish), including ventral nudging and porpoising. Ventral nudging, or one fish bumping into another, has been observed for Atlantic tarpon (*Megalops atlanticus*, Baldwin and Snodgrass 2008) and common snook (Lowerre-Barbieri et al. 2003), and suggested to be related to courtship. Porpoising or breaching, which is when individuals fully or partially jump out of the water, has also been observed for fishes and marine mammal, and often hypothesized to be related to a range of motivations, including predator avoidance (de Lima Filho et al. 2012), feeding (Martin et al. 2005; Curtis and Macesic 2011), overcoming physical barriers (Banks 1969), cooperative hunting (Klimley et al. 1996), removal of ecoparasites (Compagno 1984), and courtship and mating (Klimley et al. 1996). Even so, the only direct account of mating-related breaching for a teleost fish was observed for leopard grouper (*Mycteroperca rosacea*) with fish during a horizontal spawning rush ascending in the water column from 3 to 8 m and breaking the surface following the release of gametes (Erisman et al. 2007). Details of these reported behaviors, including their context, are different than the porpoising behaviors observed for *A. vulpes* by Danylchuk et al. (2011). Specifically, Danylchuk et al. (2011) also observed bubbles emerging from the aggregation synchronous to when porpoising was occurring, which happened for a 30–60 min period as the fish began moving offshore. Following these behaviors, tracking and telemetry data showed that these bonefish indeed moved towards a deep-water drop-off (>1000 m), and within days tagged fish were detected back in the shallow coastal flats

where bonefish are typically found (Danylchuk et al. 2011). Danylchuk et al. (2011) also observed these movement patterns and behaviors during multiple times within the putative spawning season as well as across years at the same location.

Given the unique context of observations made by Danylchuk et al. (2011), understanding the ubiquity of these putative pre-spawning behaviors could provide important clues that could be used as confirmation that specific bonefish aggregations are associated with spawning. For this study, local fishing guide knowledge to direct us towards other locations in The Bahamas where large aggregations were seasonally observed away from their typical shallow water flats (see Adams et al. 2018). We then used surface, underwater, and aerial observations to document whether ventral nudging and porpoising behaviors occurred at these locations, as well as manually tracked acoustic tagged bonefish to confirm their movement offshore at dusk. Confirming the ubiquity of these patterns and further quantifying the specific behaviors can only validate their potential as indictors of spawning areas, as well as shed light on the specific reproductive biology of bonefish.

Methods

Study sites

Field observations and tracking were conducted in The Bahamas between December 2013 and January 2016. Prior to the fieldwork, ad hoc interviews and information sharing were conducted with fishing guides, with the intent to identify as many potential bonefish pre-spawning aggregation sites as possible. Given that detailed observations and tracking of fish would occur from before dusk and into the night, final site selection was contingent upon the relative ease of logistics and safety. Site selection was also based on receiving multiple independent reports about large aggregations of bonefish during the late fall, winter, and early spring, in atypical habitats, especially locations adjacent to deep water (see Adams et al. 2018).

Two locations were selected, one on Abaco Island and another on Andros Islands. To protect the bonefish populations from potential overexploitation and disturbance, either via catch-and-release recreational angling or through subsistence harvest, we are purposely not disclosing the exact locations of these bonefish aggregation sites. Instead, only general area names will be used to reference the sites moving forward. For Abaco, the Cross Harbour (CH) site was visited 12–17 November, 2013 (full moon), and 03–10 December 2014 (full moon). For Andros, the South Andros (SA) site was visited 20–26 January, 2016 (full moon).

Behavioral observations

At each location, we conducted ad hoc visual surveys from boats and using small, unmanned aerial vehicles (UAVs, custom built and DJI Phantom 3 Pro, DJI Innovations, Shenzhen, China) to first determine the presence of bonefish aggregations. If located, we slowly approached the aggregation by boat and then snorkelers were deployed to make in-water observations. In-water surveys consisted of snorkelers staying at least 30 m away from any aggregation and floating motionless to make initial observations. Depending on the disposition of the aggregation, the snorkelers either slowly approached at approximately 5 m intervals, or remained still, especially if the fish within the aggregation moved on their own towards snorkelers. Snorkelers remained in the water following the aggregations until the sun set or if conditions became unsafe.

Snorkelers used a combination of sport cameras (Hero 3+, Hero 4, Go Pro Inc., San Mateo, CA, USA) and a video camera in underwater housing (Canon VIXIA HFS11, Tokyo, Japan, with Equinox HD6 housing) to record the behaviors of the aggregation and individual bonefish. We opted not to make observations on SCUBA because of concerns related to disturbing the aggregation, either from bubbles or being perceived as a predator or threat. Depending on surface conditions, water clarity, light availability, and distance from the aggregation, snorkelers continuously recorded video, intermittently recorded video, took still images, or just made ad hoc visual observations of the aggregation. When conditions permitted, additional UAV surveys were also conducted.

On location, digital video and images were downloaded and reviewed daily, and then stored on multiple hard drives. Digital images were viewed by multiple individuals to confirm whether the behaviors documented by Danylchuk et al. (2011) were observed. Video footage was viewed at full speed and half speed to allow for viewing individual behaviors, especially when bonefish were in dense aggregations that were

constantly moving. Once confirmed, video footage were used to quantify the frequency at which the behaviors occurred. For particular behaviors, video footage was imported into ImageJ to better facilitate counting (manually) and to quantify the orientation of fish. For porpoising, we measured the approximate angle of ascent in relation to the water's surface, whether the fish fully or partially breached the surface, and the angle of descent back to the aggregation. For ascent and descent angles, given that the water's surface was dynamic, three separate measures were determined for each fish and then aggregated as a mean.

Manual tracking

Manual tracking was conducted using a mobile acoustic receiver with a directional hydrophone (VEMCO VR-100, Amirix Inc., Shad Bay, NS, Canada) and continuous transmitters with depth sensors (V9P 2H, 9 mm diameter, 21 mm in length, 1.6 g in air, 2000 ms transmission period, pressure sensor limit 50 m). Bonefish were caught via hook and line or via cast net from the aggregation, and transmitters were quickly inserted into the stomach following Danylchuk et al. (2011). Specifically, the continuous transmitter was fitted into the end of a smooth plastic tube that was gently inserted into the esophagus. Once in the stomach of the bonefish, a plunger inside the tube was used to release transmitter, with the entire procedure taking <30 s. Fork length (mm) and sex (release of gametes when males were gently palpated or females cannulated, see Adams et al. 2018) was determined for each fish. Following gastric implant, bonefish were held in a plastic tote (35 × 61 × 25 cm) for approx. 3 min in the event the tag would be regurgitated, and then the fish was released back into the aggregation. Once at large, we manually tracked tagged bonefish from a boat while drifting 30–50 m from the aggregation or slowly following the aggregation as it moved. Tracking was done continuously unless conditions became unsafe for boating or if we lost track of the tagged fish and had to broaden our movements to relocate them.

Data from manual tracking were downloaded from the VR100, and used to map the movement patterns and depth profiles of the tagged bonefish. Mapping of the movement patterns was done using Google Earth Pro, and individual variation in depth profiles analysed using univariate statistics in JMP Pro (version 13.0.0; SAS Institute Inc., Cary, NC, USA).

Results

Locating aggregations & aggregation behavior

Large aggregations of bonefish (approx. 2000–5000+ individuals) were located on both Abaco and Andros Islands at locations revealed by local guides, and around the full moon periods. On Abaco, the same location was visited in two consecutive years (November 12–17, 2013; December 3–10, 2014), and each time large bonefish aggregations were located. Only boat and snorkelling surveys were used on Abaco, as weather conditions and equipment issues prevented the use of a UAV for aerial surveys and imagery. The aggregations on Abaco were initially spotted in approximately 2 m of water over mixed hard bottom habitat adjacent to a point of land, and then observed moving into 8–10 m water with sand bottom and small coral heads. Conversely, on Andros (January 20–26, 2016), both topside boat and UAV surveys were used to find bonefish aggregations, while the UAV was used to scan adjacent habitats and larger areas of the shoreline for other schools of bonefish. The aggregation on Andros was found in a dredged channel, approximately 5 m deep. Aerial surveys were able to identify the bonefish aggregations, especially in locations where the benthos was relatively light (i.e., sand), water relatively shallow (>5 m), when cloud cover was minimal, and when the aggregation was not moving rapidly (Fig. 1).

In all cases, bonefish within the aggregations moved in a circular rotation around a central core, either in a clockwise or counter clockwise direction (See YouTube

Fig. 1 Oblique aerial image of a bonefish aggregation (UAV elevation, 25 m) in The Bahamas

video - https://www.youtube.com/watch?v=RYytheGXEr4). The compactness and overall shape of the aggregations differed temporally, beginning more dispersed and oblong and then becoming more compact and resembling a 'baitball', similar to what is often seen for pelagic fishes such as sardines. The shape of the aggregations also changed when a shark, great barracuda, or even a sea turtle approached, with the aggregation either quickly spreading out and re-condensing, or creating a halo of bonefish around the transient animal. The diversity and abundance of potential predators, such as sharks, great barracuda, and large snappers and groupers, differed daily when observing the aggregations, as well as among aggregation sites. The extremely large aggregation we observed on Abaco had large Cubera snappers (*Lutjanus cyanopterus*) occasionally rising from coral heads below as well as a solitary large barracuda drifting by, but sharks were only observed one of the 2 years. On Andros, we observed as many as three Caribbean reef sharks (*Carcharhinus perezii*) occasionally passing by or through the aggregation, and local fishers reported also seeing bull and hammerhead sharks in this location at other times. Of note is that the aggregations behaved differently when approached by a potential predator versus when a snorkeler slowly approached or if the aggregation moved towards a snorkeler on their own, thus allowing us to obtain rather detailed images and video of their behaviors, particularly when water clarity was good. Interestingly, even when we observed potential predators, we did not observe a predatory encounter on bonefish in any of the aggregations.

Bonefish behavior in aggregations

We observed porpoising behavior at both locations prior to sunset, just before or while the aggregations began moving offshore (See YouTube video - https://www.youtube.com/watch?v=3NXFXa_dnUw). The most vigorous porpoising was observed during the survey on Abaco in 2013, with bonefish emerging from an aggregation of well over 5000 fish (Fig. 2a). Porpoising at the Abaco location in the following season was not as prominent, likely masked by choppy surface conditions. During the time of our second survey on Abaco, the bonefish aggregation was also considerably smaller (approx. 2000 fish) and the number of potential predators, particularly sharks, was higher. For the Andros location, porpoising behavior occurred over a more protracted timeframe, beginning sporadically around 15:00 h, but intensifying at approximately 17:30 h, just as the sun began to set.

In all cases, porpoising was episodic (i.e., not continuous) and displayed by bonefish emerging from the top of the aggregation (Fig. 2b). Although single fish were observed porpoising, it was more common that episodes of multiple bonefish (2–200 individuals) occurred lasting from 3 to 65 s. In most cases, the frequency of porpoising bouts increased as the sun approached the horizon and as the aggregation began to move offshore, with the total duration of observed sequential bouts occurring over approximately 20 min.

Bonefish that were observed porpoising ascended from the aggregation at a mean angle of 28 ± 8° SD (Fig. 2b) and broke the surface head first, either jumping partially or fully out of the water (Fig. 2c). In all cases, at least the portion of the fish anterior of the dorsal fin emerged from the water, exposing the mouth and operculum (Fig. 2d). Bonefish that partially emerged tended to land ventrally on the surface ('belly flopped') and then descend, while bonefish that fully emerged tended to land snout first; in both cases fish descended back towards the aggregation at a mean angle of 39 ± 12° SD. There was a significant difference between the angle of ascent and descent (t-test, $t = 6.26$, $df = 242$, $p < 0.001$). We attempted to determine whether the same individuals were porpoising sequentially, however this was not possible using the video footage because the aggregations were too dense to follow individual fish for more than 30 s.

In situ observations and video footage showed bubbles emerging from the bonefish aggregations (Fig. 3), but only during or shortly following bouts of porpoising. Reducing the speed of video playback showed that the bubbles were originating predominantly from the opercula. Because of the orientation of the bonefish and angle that the video was captured, it was difficult to determine whether bubbles were also emerging from the vent.

Detailed scan sampling of over 180 min of video files from both locations revealed only three individual accounts of ventral nudging. For the video footage with the greatest water clarity (Abaco in 2013), the bonefish aggregation was large and very dense, making it difficult to observe behaviors of bonefish that were not swimming along the perimeter of the mass of fish.

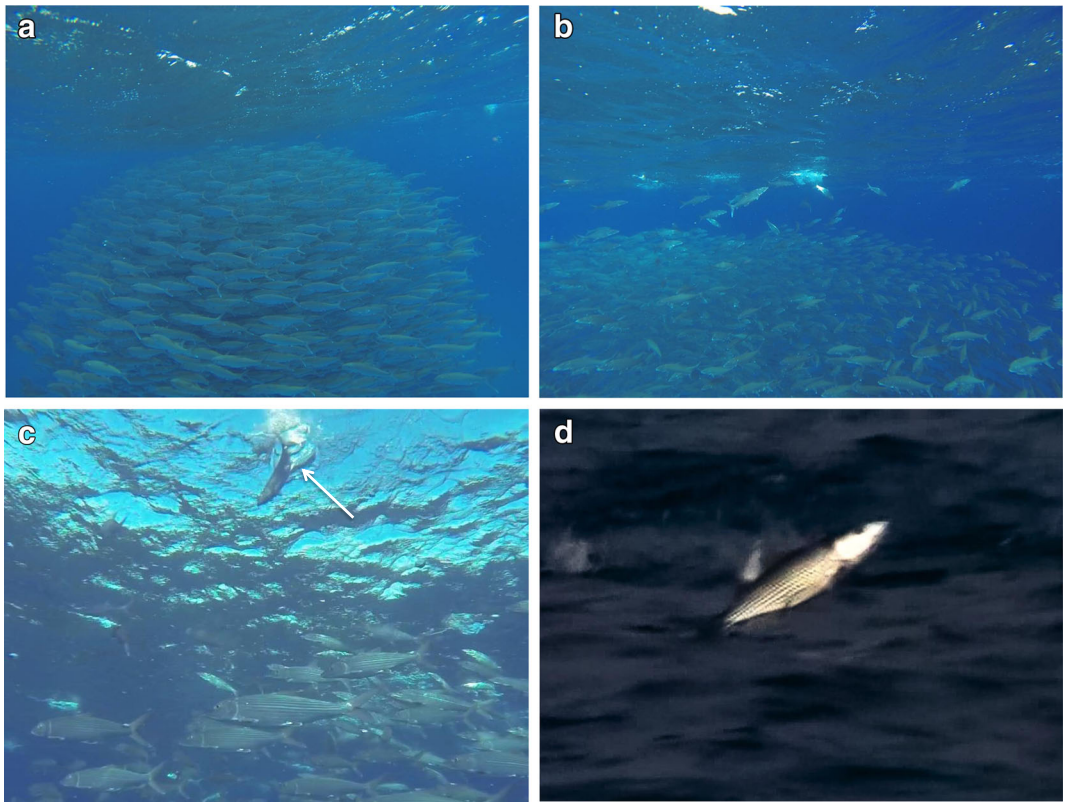

Fig. 2 **a** Aggregation of bonefish in approximately 10 m of water, as it moved parallel to shore, near where the island shelf meets a deep-water drop-off; **b** Bonefish rising from the aggregation, porpoising, and descending back to the aggregation; **c** a bonefish re-entering the water after porpoising (as indicated by white arrow); and **d** a bonefish porpoising with the entire body above the surface of the water

Offshore movement patterns

For both Abaco and Andros locations, bonefish were gastrically tagged as soon as possible after we identified a large prespawning aggregation. On Abaco, two female (430 ± 28 mm FL) and three male (409 ± 31 mm FL) bonefish were gastrically tagged in 2013, and two female (450 ± 42 mm FL) and one male (390 mm FL) bonefish in 2014, while on Andros two female (448 ± 18 mm FL) and two male (433 ± 11 mm FL) were tagged in 2016.

On Abaco, in 2013 and 2014 the aggregations moved relatively slowly (approx. 2 km/h) parallel to shore along the 8–10 m contour, with the aggregation remaining between the surface and 5 m in depth during the mid-afternoon, and then turning slightly seaward at approximately 17:00 h toward where the reef shelf met a deep-water drop-off approximately 100 m further offshore. The onset of offshore occurred within 20–40 min after sunset. On Andros, at 17:44 h the aggregation moved almost perpendicular to shore and towards the outer reef and drop-off of the Tongue of the Ocean. We were able to track three of the gastrically tagged

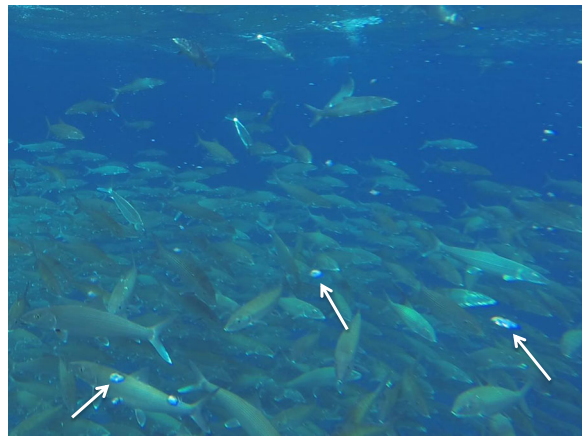

Fig. 3 Bubbles (as indicated by the white arrows) rising from a bonefish aggregation

bonefish for over 1.5 km as they moved from the pre-spawning aggregation site along a dredged channel to just before the barrier reef edge. These fish covered this distance in 44 min (approximately 2 km/h), however rough seas and darkness prevented us from staying close to the aggregation beyond the barrier reef.

For Abaco in 2013, we were able to follow the aggregation for nearly 10 h as conditions were calm and our boat could remain close to the transmitters' signals. Approaching sundown, snorkelers periodically verified that we were following the aggregation, however they ceased doing so once it was dark; we did not want to use dive lights in the event it disrupted the aggregation. Simultaneously, we used the directional hydrophone to manually track a single bonefish that was in the aggregation (female, 450 mm FL), and followed it as it moved off the edge of the drop-off. Depth detections from this transmitter indicated that it descended from 5 m to greater than 50 m between 17:38 and 18:17 h (at a rate of 0.02 m/s; Fig. 4). We could not determine the final depth this bonefish had reached because the depth surpassed the 50 m depth limit of the sensor. Between 20:17 and 20:30 h the bonefish quickly ascended from >50 to 25 m, at a rate of 0.32 m/s, after which we followed the bonefish along the edge of the drop off as it slowly moved back into shallower water at a relatively consistent depth. Water past the edge of the drop off exceeded 1000 m in depth. Unfortunately, tracking ceased when the battery in the manual hydrophone expired at 22:04 h. We returned to the aggregation site the following morning (06:57 h) and could not relocate any of the tagged bonefish. On Abaco in 2014, we manually tracked three individuals in an aggregation that moved towards the edge of the drop off near close to where the aggregation moved offshore in 2013, however a combination of strong winds, rough seas, and boat issues stopped us from following these fish beyond the drop-off into open water.

Discussion

While attempting to observe and track fish at dusk and into the night, on each occasion we observed sizable, dense bonefish aggregations moving away from the shore towards deep-water drop-offs during full moon periods in winter months. At each location, we also observed multiple individuals porpoising at dusk just prior to or during the transition offshore – a behavior not observed for bonefish when they are found on shallow

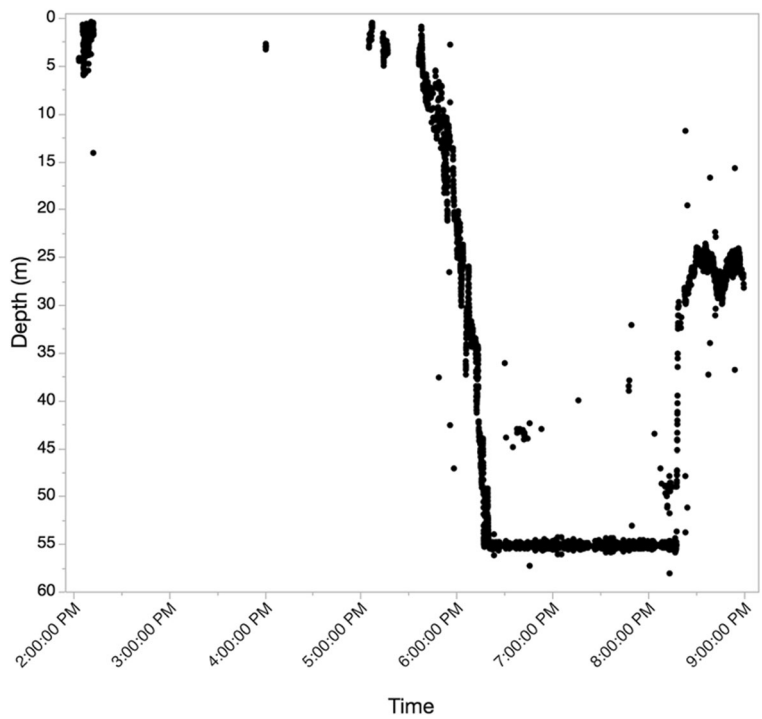

Fig. 4 Depth profile for a female bonefish (450 mm FL) as it moved in an aggregation from inshore to beyond the edge of a deep-water drop-off. Note that tag sensor limit was 50 m

flats (see Adams et al. 2018). These repeated observations are consistent with the criteria provided by Domeier (2012) regarding the identification of spawning aggregations, as well as with observations made by Danylchuk et al. (2011) for bonefish on a different island in The Bahamas. Even though actual spawning bouts were not observed, these repeatable patterns of aggregations and behaviors could be used to help characterize locations that are critical for the reproductive life history phase of bonefish, especially when combined with other information such as traditional ecological knowledge, larger scale tagging efforts, and biological sampling (e.g., oocyte histology) (Adams et al. 2018). As seen with other fish species that aggregate to spawn, identifying and protecting such locations should in most cases reduce the risk of overharvest and habitat loss, and thus be of considerable conservation value (Sala et al. 2001).

The most obvious and predictable behaviors were that they moved offshore at dusk (independent of the daily tidal cycle) and some individuals in the aggregations were porpoising. Danylchuk et al. (2011) postulated that porpoising behavior could be for gulping air to fill their swim bladders as a way to increase buoyancy prior to moving into deep water, where they potentially spawn near the surface of the water column. In their study, Danylchuk et al. (2011) tagged a small number of bonefish with coded tags equipped with depth sensors and detected an individual at 5.7 m on a fixed acoustic receiver moored in 26 m of water at the edge of the drop off of the Exuma Sound. This is similar to what we recorded on Abaco when manually tracking one individual in a large aggregation as it moved towards a deep-water drop off; however, once at the drop off this bonefish descended to a depth of over 50 m and then ascended rapidly, all within a 3 h period. Even though this was quantified for just one bonefish, the pattern resembles the 'spawning rush' behavior displayed by aggregating broadcast spawners, such as groupers (Peta et al. 2005) and snappers (Carter and Perrine 1994), when males chase females up into the water column where both release their gametes for fertilization (Johannes 1978). Perhaps, given that porpoising occurred as the aggregation moved offshore, and that bonefish are physostomus, any air trapped in the swim bladder and potentially the gastrointestinal track would be compressed with increasing water depth. In fact, a female bonefish (430 mm FL) captured from the aggregation on Andros that died (after an attempt to induce ovulation via hormone injections) and subsequently dissected had a fully inflated swim bladder measured to be approximately 172 cc (19 cm long × 3.5 cm in diameter). If this individual moved offshore and descended past 50 m, the swim bladder would compress to 5 cc, allowing for 167 cc of abdominal space for expansion of oocytes as they hydrate. If female bonefish are hydrating their eggs as they descend (see Adams et al. 2018), then the expansion of air could help force the eggs out of the gonoduct (i.e., 'pneumatic assist') during a rapid assent, potentially when males are releasing their sperm. This hypothesis is also supported by observations from *A. glossodonta* in French Polynesia that are harvested when they migrate from inshore pre-spawning aggregations and also when returning to the flats, with the former having fully inflated swim bladders and the later deflated swim bladders (A. Filous, unpubl. data).

Other hypotheses for the evolution of porpoising behavior are that it is a form of male courtship display (Molloy et al. 2012), or that gulping air and the release of bubbles is an anti-predator behavior especially when the large aggregation moves into deeper water at night (Nøttestad 1998). Regardless of its purpose, the predictability of porpoising when large aggregations move offshore suggests that it is related to spawning, yet clearly more work is needed to understand this behavior. Increased sampling (capture and dissection) of bonefish immediately before and after they move offshore, descend to considerable depth, and then return to shallow water may provide additional insights into the purpose of porpoising, especially if sampling reveals that only females porpoise.

Group breeding and associated behaviors have adaptive benefits for adults, ranging from increased mate-encounter rates, increased reproductive output, and reduced predation rates on adults and resulting fertilized eggs (Molloy et al. 2012). Although aggregations can increase conspicuousness to predators, we did not directly observe potential bonefish predators (sharks, barracuda, large snapper or groupers) actually preying on any fish in an aggregation. Nevertheless, once the sun set, predator-prey dynamics at the aggregations could have changed with predators becoming more aggressive as the ability of bonefish to see decreased. In fact, during our work on Abaco in 2014, there was a distinctive fishy odour in the air after the sun set, which could have been related to bonefish being preyed upon. Fishy odours during aggregations could also result from the release of oils related to spawning activity or other biological activity.

Although it is challenging to directly evaluate the costs and benefits related to the evolution of spawning aggregations in fish (Molloy et al. 2012), the fact that spawning aggregations and associated behaviors can be conspicuous and occur at discrete times and locations may increase the potential to structure management strategies to protect this critical life stage. For *A. vulpes*, the predictable nature of aggregations and porpoising behavior could be used to locate seasonal spawning areas throughout The Bahamas, the Florida Keys, and the Caribbean. These traits could also be used to compare the reproductive ecology of *A. vulpes* to that of other species of bonefish that occur in the Indo-Pacific. Given their economic value as a sportfish, developing countries with limited funding and resources for detailed tagging and telemetry studies could rely on visual observations of aggregations as well as limited biological sampling to confirm the location of bonefish spawning aggregation sites, and afford these locations the appropriate protection.

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