DEVELOPING SPAWNING PROTOCOLS AND EMBRYOLOGICAL BENCHMARKS FOR A TROPICAL MARINE FISH (*Albula* spp.) IN CAPTIVITY

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Paul Wills, Department of Environmental Science, and has been approved by the members of his supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

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Relying on field research to complete the life history for certain fish species can be inadequate, but laboratory research can be used to fill these gaps. These gaps exist for Bonefish (*Albula* spp.), a tropical marine fish and popular sportfish. In this study, aquaculture techniques were applied to Bonefish in a captive setting at Harbor Branch Oceanographic Institute (HBOI) and Cape Eleuthera Institute (CEI) to induce spawning and describe early ontogeny. Photothermal manipulations and hormone injections were used to induce gonad maturation and spawning, which was achieved once at CEI and is the first record of hormone-induced spawning for Bonefish. From that spawn, egg and larval development were recorded and described through 26 hours and 56 hours respectively, representing the first record of these early life stages for Bonefish.

This work expands upon what is known about Bonefish reproductive biology and will be useful for management and future captive research.

DEDICATION

I dedicate this thesis to my mother, Theresa, who instilled in me a lifelong love of learning, and my girlfriend, Colleen, whose patience and support during my project have meant everything.

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INTRODUCTION

Anthropogenic activity threatens coastal habitats around the world (Valiela et al. 2001, Orth et al. 2006, Polidoro et al. 2010, Short et al. 2011). With 39% of the world population residing within 100 km of a coastline (Kummu et al. 2016), increases in development, industry and agriculture continue to degrade or to destroy these coastal habitats. For example, in Florida, populations have risen from 752,619 in 1910 (U.S. Census Bureau 1913) to 18,801,310 in 2010 (U.S. Census Bureau 2012), a nearly 2,500% increase. Nearshore habitats (e.g., seagrasses, mangroves) suffered due to this increase. A large seagrass die-off in Florida Bay occurred in the late 1980s, resulting in the loss of *Thalassia testudinum* (28%), *Halodule wrightii* (88%), and *Syringodium filiforme* (92%) standing crop (Hall et al. 1999). Strong and Bancroft (1994) examined aerial coverage of mangrove forests in the Florida Keys between 1945-1959 and 1991 and they found that islands accessible by road lost 19.2% of their mangrove forests, compared with islands only accessible by boat, which lost only 0.2%, mean forest size decreased by 15%, and the remaining forests were fragmented. Fishes use both seagrasses and mangrove forests as nurseries and foraging grounds. The degradation and loss of these habitats causes population decline through increases in recruitment failure and mortality (Jackson et al. 2001, Manson et al. 2005).

The Bonefish (*Albula vulpes*) is a marine fish species that inhabits shallow, coastal waters throughout the Caribbean basin, including southern Florida (Crabtree et al. 1998, Humston et al. 2005, AJ Danylchuk et al. 2007, Vásquez-Yeomans et al. 2009). Both seagrass and mangrove habitats may act as nurseries for juvenile Bonefish as well as providing foraging opportunities and refuge from predators to both juveniles and adults (Adams et al. 2014). Bonefish populations decline as these habitats and their services are lost (Adams et al. 2012, Adams et al. 2014). Tag-recapture and acoustic telemetry studies show that adult Bonefish have small home ranges and exhibit high site fidelity (Humston et al. 2005, Murchie 2010, Larkin 2011), further increasing their susceptibility to declines in habitat quality or quantity.

Habitat connectivity to pre-spawning and spawning sites, which Danylchuk et al. (2011) showed were offshore, can also be disrupted by anthropogenic activity. Johannes and Yeeting (2001) documented in Kiribati that causeway construction restricted passes historically used by Shortjaw Bonefish (*A. glossodonta*) for spawning migrations out of lagoons. However, there is some evidence that connectivity can be restored after disruption. Murchie et al. (2015) used telemetry in Grand Bahama to show that Bonefish were capable of using the Grand Lucayan Waterway – a man-made canal installed to replace the severed Hawksbill Creek – for spawning migrations from the north to the south side of the island.

In addition to habitat disruption, Bonefish populations also experience pressure from recreational and, in some countries, artisanal fisheries (AJ

Danylchuk et al. 2007, Murchie 2010, Larkin 2011). Bonefish are an important sportfish to recreational anglers due to their renowned fighting ability, relative to their size, when hooked (Kaufman 2000, Fernandez 2004). Bonefish support economically important recreational fisheries in Florida, Belize, and The Bahamas. Bonefish are part of a recreational flats fishery with an annual economic impact of $465 million/year in Florida (Fedler 2013), $56 million/year in Belize (Fedler 2014) and $141 million/year in The Bahamas (Fedler 2010). Bonefish are part of subsistence fisheries in the Caribbean as well (Clark and Danylchuk 2003), although these fisheries have been declining in the region (Rudd 2003). Increased fishing pressure may present further threats to Bonefish sustainability (Larkin 2011).

No formal stock assessments have been conducted for Bonefish in Florida. Scientific surveys (Ault et al. 2008, Larkin 2011), interviews with long-time guides and anglers in the Florida Keys (Frezza and Clem 2015), and fishing logbook analyses from Florida Bay (Santos et al. 2017) indicate declining populations, possibly as much as 90% (Curtis 2004). The survey by Ault et al. (2008) estimated there were 364,000 fishable Bonefish (i.e. ≥35 cm) in the entire Florida Keys. However, little is known about juvenile *A. vulpes*, which were excluded from the survey, or the habitats they utilize, in the Florida Keys. Adams et al. (2007) caught juveniles in the Florida Keys, along sandy, windward beaches. However genetic testing identified 93.97 % of the juveniles as *A. goreensis*, a morphologically identical species to *A. vulpes*. In Eleuthera, The Bahamas, Szekeres et al. (2017) did find juvenile *A. vulpes* in shallow, protected

bays. Bays with similar morphology were surveyed in the Florida Keys, but did not contain juvenile *A. vulpes* (B. Black, Bonefish & Tarpon Trust, personal communication). Without recruitment data, any Bonefish stock assessment is incomplete. However, the decreasing trend in overall population and lack of juveniles found in the Florida Keys indicate the need for more conservative estimates. New threats, particularly loss of habitat area and quality, combined with an incomplete knowledge of *A. vulpes* life history and population dynamics, led the International Union for Conservation of Nature (IUCN) Red List to classify Bonefish as Near Threatened across its range, with localized Vulnerability in the Florida Keys (Adams et al. 2012).

It is critical to keep the Bonefish population above the minimum viable population (MVP), which is the number of individuals within a population required to persist through events capable of causing extinction (e.g., stochasticity, disturbances) (Shaffer 1981). MVP is measured as the probability of a population persisting for a specific length of time (Shaffer 1981). When a population gets below the MVP, the probability of persistence decreases and leads to population extinction. Small populations and populations with limited geographic ranges are more susceptible to extinction (Traill et al. 2007) and MVPs are a useful tool for determining the probabilities of these populations going extinct. Establishing minimum viable populations should be done for each species, considering its life history. Estimates range from 4,000 to 7,000 (Reed et al. 2003, Traill et al. 2007) for terrestrial vertebrates. However for fishes, where fewer MVP estimates have been conducted, the estimates range from 10,000 to 20,000 in Chinook salmon

to 500,000 in Herring or Anchovy (Traill et al. 2007). An MVP has not been calculated for Bonefish because of lack of information about spawning frequency, larval survival, and recruitment. Without this knowledge, managers should prioritize sustaining current populations and work towards rebuilding them to insure Bonefish persistence in the Florida Keys. A broad strategy should be used for restoring Bonefish populations, including understanding the causes of population decline, stopping the decline, and aiding population recovery.

One way to learn more about Bonefish life history is through research on captive fish, including spawning and rearing. Not only would this method provide new information about Bonefish life history, but a successful spawning program would also provide an additional tool (i.e. stock enhancement) within the restoration strategy for Bonefish. Captive spawning research, done concurrently with the identification of the proximate causes of population decline in Florida, will provide the knowledge necessary to prevent further declines in Bonefish populations. Ideally populations would recover naturally, but this may take longer than anticipated (Hutchings 2000). Stock enhancement provides a tool to accelerate a natural recovery.

Fish aquaculture techniques have been used for over a century in the United States (Green 1870). Aquaculture has primarily been used for commercially or recreationally fished species to provide an alternative to overexploited stocks or rebuild collapsed stocks (Diana 2009, Ireland et al. 2002). Bonefish would benefit from the development of a culture model because of population declines in the Florida Keys, and their recreational, economic and

ecological value. Working with Bonefish in captivity would also grant valuable time to work with adult, larval, and juvenile fish in a lab setting as opposed to the field work conducted thus far (e.g., Snodgrass et al. 2008, Vásquez-Yeomans et al. 2009, Danylchuk et al 2011). New knowledge from captive efforts will also assist with the identification of potential causes for population decline and help prevent further population declines in the Florida Keys. By combining the knowledge gained from field work with research in a captivity, scientists and managers will achieve a greater understanding of Bonefish.

To develop a successful culture protocol, a review of *A. vulpes* life history is necessary. Despite first being described in 1758 by Linnaeus, much of what is known about Bonefish life history and biology has only been determined in the past few decades. Bonefish use several distinct habitats throughout their life: benthic, neritic, intertidal, subtidal, and oceanic (Humston et al. 2005, Danylchuk et al. 2011, Adams et al. 2014). Adult *A. vulpes* are found in shallow water along coastal flats and tidal creeks, but also use deeper channels as refuge from cold temperatures (Humston et al. 2005, Murchie et al. 2009). Telemetry and tag-recapture studies indicate that adult Bonefish occupy small home ranges, roughly 0.5 km^2 (Humston et al. 2005, Boucek et al. 2018).

Bonefish play an important ecological role in shallow coastal environments. Bonefish are meso-predators, consuming primarily benthic invertebrates – shrimp, bivalves, and polychaete worms – and small fishes (Colton and Alevizon 1983, Crabtree et al. 1998, Snodgrass et al. 2008). Benthic feeding results in bioturbation, impacting oxygen content, infaunal communities,

biogeochemical cycling, and microbial communities in these habitats (Mermillod-Blondin 2011, Laverock et al. 2011). Sharks and barracuda prey on Bonefish (Cooke and Philipp 2004, SE Danylchuk et al. 2007), linking them to higher trophic levels. Offshore spawning (Danylchuk et al. 2011) and an extended pelagic larval stage (Mojica et al. 1995) also link Bonefish to the oceanic environment.

Bonefish can live for up to 20 years (Larkin 2011) and grow to larger than 70 cm (Crabtree et al. 1996). For currently unknown reasons, Bonefish in the Florida Keys grow three times faster than Bonefish in the Caribbean Sea, but mature at the same ages (Crabtree et al. 1997, Adams et al. 2007). Bonefish in the Florida Keys are reproductively active from November to May and take 3 to 4 years to mature, with males maturing earlier and at smaller sizes (3.6 years, 418 mm FL) than females (4.2 years, 488 mm FL) (Crabtree et al. 1997). Total fecundity in Bonefish from the Florida Keys ranges from 0.4-1.7 million oocytes per fish and is positively correlated with weight. Relative fecundity ranges from 159 to 385 oocytes/g and is positively correlated with age (Crabtree et al. 1997).

During spawning, Bonefish in The Bahamas undergo migrations of up to 110 km (Boucek et al. 2018) to specific pre-spawning sites, where they form large aggregations (Danylchuk et al. 2011). The migration and spawning in The Bahamas occurs around the full and new moons from October through April (Danylchuk et al. 2011). Spawning migrations have not been observed in the Florida Keys, but Crabtree et al. (1997) found Bonefish were reproductively active from November to May. Commercial fishermen in the 1930s may have

encountered a pre-spawn aggregation in the Florida Keys (Rowles 1959), but no other occurrences or verified aggregations in the Florida Keys have been reported since then.

Based on known locations of Bonefish pre-spawning aggregations in The Bahamas and Kiribati (A. Adams, personal communication, Johannes and Yeeting 2001), the preferred habitat for these aggregations is a shallow bay (10-20 m) within 1-5 km of a shelf edge, where the offshore water depth exceeds 1 km. During the pre-spawn aggregation, Bonefish gulp air and jump out of the water. These behaviors are not observed when they are on the flats and are believed to play a role in spawning, as they precede the movement of fish offshore (Danylchuk et al. 2018), where spawning occurs (Danylchuk et al. 2011). Once offshore, the school of Bonefish descends to depths exceeding 55 meters for several hours, before returning to the surface and then inshore (A. Adams, unpublished data). It is believed that egg hydration occurs during the time Bonefish are at depth. The air ingested during the pre-spawning aggregation may be compressed at depth due to increasing pressure and the drop in pressure during ascent would cause the air to expand, which may be used as a pneumatic assist for egg and sperm release.

Direct observation of spawning in the wild has not occurred however, due to rough seas limiting access to the spawning site, the occurrence of spawning at night, and the location of spawning at depth. In contrast to spawning in the wild, volitional spawning has been observed in captivity (D. Wert, personal

communication, Haley 2009). These observations were made in a large public aquarium that was 13 m deep, with shallower ledges also present.

After spawning, eggs hatch into a unique larval form, known as the leptocephalus, which is laterally compressed, transparent, and composed mainly of a gelatinous, extracellular matrix (Pfeiler 1991). The leptocephalus larval stage is a synapomorphy for the superorder Elopomorpha, which includes bonefish, tarpon, ladyfish, eels, and deep-sea eels (Wiley and Johnson 2010). Larval duration, the amount of time it takes a fish to grow from hatching to metamorphosis into a juvenile, varies widely among elopomorphs. A comparative study of freshwater eels by Kuroki et al. (2014) found that the tropical eel *Anguilla bicolor bicolor* can metamorphose after only 40 days, while *Anguilla anguilla* can take as long as 300 days. The main driver for these variations is temperature, with warmer waters resulting in shorter larval durations, and distance from spawning to the recruitment habitat, which for *Anguilla anguilla* is 6,000 km (Bonhommeau et al. 2009). *Albula vulpes* larvae, which occur in tropical water, metamorphose after 41-71 days; during this time, they grow up to 70 mm (Mojica et al. 1995).

The diet of the leptocephalus larvae in general is still a mystery. Larval feeding has never been observed in the wild for any Elopomorph species. Stable isotope studies indicate that eel leptocephali feed at a low trophic position (Miller et al. 2013). Visual gut content analyses of *Anguilla japonica* larvae found fecal pellets, appendicularian houses, and transparent exopolymer particles (Mochioka and Iwamizu 1996, Tomoda et al. 2018). In *Anguilla anguilla*, DNA gut content

analysis identified ctenophores as the dominant organism present (Riemann et al. 2010). Diet research with captive-bred eel leptocephali has had limited success. Mochioka et al. (1993) observed eel larvae ingesting squid paste. Okamura et al. (2013) used hen egg yolk mixed with krill to feed leptocephali. Shark egg yolk mixed with krill was sufficient to rear *Anguilla japonica* leptocephalus to metamorphosis (Okamura et al. 2013), but the diet causes deformities, is reliant on an endangered species of shark, and likely not what leptocephali consume in the wild. Tomoda et al. (2015) observed ingestion of transparent exopolymer particles and appendicularian houses in captivity. In Europe, a prepared rotifer paste was the most successful diet tested for *Anguilla anguilla* (Butts et al. 2016).

As they develop, leptocephali go through two phases: Phase I, when they feed and grow, and Phase II, when they cease feeding and undergo metamorphosis (Pfeiler 1999). Instead of exogenous feeding in Phase II, leptocephali use endogenous carbohydrates (glycosaminoglycans), which cause larvae to shrink (Pfeiler 1986). Upon completing metamorphosis, Bonefish larvae use incoming spring tides during new moons to move into estuaries and bays (Mojica et al. 1995).

While much of Bonefish life history is still poorly understood, enough information exists to begin research on captive spawning. Previous aquaculture research shows that holding fish in captivity can disrupt environmental cues and natural behaviors, such as spawning (Zohar and Mylonas 2001). Because of this, it is necessary to replicate natural environmental conditions (e.g., temperature,

photoperiod, salinity) in the captive environment. In some species (e.g., *Sciaenops ocellatus*), this is all that is needed to induce gametogenesis and spawning (Chamberlain et al. 1987). However, replicating environmental conditions is not always sufficient for gametogenesis and volitional spawning. Some species must be induced to form gametes and to spawn with the aid of exogenous hormones (Zohar and Mylonas 2001, Mylonas and Zohar 2007).

While no previous work has been done on Bonefish, there are efforts in Europe and Japan to culture eels in captivity (Ohta et al. 1997, Ahn et al. 2012, Sørensen et al. 2016). These efforts have failed to produce volitional spawning and have relied upon hormone-induced spawning (Zohar and Mylonas 2001, Mylonas and Zohar 2007). Commonly used hormones for eels are carp pituitary extract (CPE) or salmon pituitary extract (SPE) (Ohta et al. 1996b, Palstra et al. 2005) and human chorionic gonadotropin (HCG) (Ohta et al. 1996b, Palstra et al. 2005). Gonadotropin-releasing hormone agonists (GnRHa) are another hormone type used successfully for captive spawning of other fishes (Forniés et al. 2001, Dimaggio et al. 2014). Both hormone types work at different levels of the hypothalamus-pituitary-gonad axis, which is responsible for controlling reproduction. GnRHa simulates the effect of a reproductively active hypothalamus. The hypothalamus regulates the release of gonadotropins (Gt-I and Gt-II) from the pituitary. Pituitary extracts already contain these gonadotropins when injected and mimic a reproductively active pituitary. These gonadotropins then act on the gonads to control gametogenesis (Harvey and Carolsfeld 1993). 17α, 20β-Dihydroxy-4-pregnen-3-one (DHP) is a maturation

inducing hormone, now frequently used in the final stages of oogenesis for induction of final oocyte maturation (FOM) and ovulation in several eel species. (Ohta et al. 1996b, Lokman and Young 2000, Pedersen 2003). Because Bonefish and eels are elopomorphs, the successful culture of eels using hormones suggests that similar results can be achieved with Bonefish, despite differences in spawning behavior.

Successful fish culture is not just dependent on conditioning and spawning in captivity. Spawns must produce viable gametes, resulting in fertilized eggs that develop properly. Thus, after completing a spawn in captivity, eggs must be checked for cell division, which indicates fertilization. Fertilized eggs are then incubated and hatched under natural or ideal conditions. Monitoring the development of the embryo and larva provides direct metrics to quantify the success of a spawning event. Recording developmental stages and when they occur, along with photographing those stages under known conditions provides a valuable reference for future culture efforts. As the embryologic development of Bonefish has never been described before, collecting these data also provides a critical piece of missing information about Bonefish life history. This time series has been conducted on other elopomorphs (Lokman and Young 2000, Ahn et al. 2012, Sørensen et al. 2016), providing some examples of what can be expected for Bonefish. Having a better understanding of these critical early life stages will allow for optimization of culture protocols for higher quality gametes, eggs, and proper development.

The Bonefish Research and Restoration Program (BRRP), based at Florida Atlantic University-Harbor Brach Oceanographic Institute (FAU-HBOI), was started in 2016 to learn more about Bonefish spawning while in captivity. Development of a successful culture protocol for Bonefish is a main goal of the BRRP. Fitting into this larger goal, the specific objectives of this thesis are 1) to condition and induce spawning in Bonefish (*A. vulpes*) in captivity, with the use of hormones if necessary, and 2) develop a time series of the embryologic and larval life stages of Bonefish. These research objectives are crucial first steps towards partially achieving the goals of the BRRP.

CHAPTER 1 – CAPTIVE SPAWNING

Introduction

Captive breeding of fishes presents unique research challenges as replicating the conditions that induce gonad development and spawning can be complicated and difficult to recreate in captivity (Zohar and Mylonas 2001). Despite these difficulties, working with fish in captivity is a common technique for research into fish biology, management, or culture (Tanaka et al. 2003, Lorenzen et al. 2012) especially as wild populations of many marine species decline (Hutchings 2000, Hutchings and Reynolds 2004). Holding fish in captivity allows for fine-scale manipulation of environmental parameters, the administration of reproductive hormones, and the ability to conduct long-term observations without having to recapture fish in the wild. Much of the research to date on captive fish spawning has primarily focused on temperate, freshwater species (Araki et al. 2007, Yaron et al. 2009, Steeby and Avery 2005). For marine species, there is a large body of research for hormone-induced captive spawning of Japanese and European Eels (*Anguilla japonica* and *Anguilla anguilla*), both of which have complicated life cycles, support commercial fisheries, and are experiencing population declines (Ahn et al. 2012, Butts et al. 2016). Commercial efforts for the aquarium trade have included captive spawning of marine ornamental fish, however, much of these efforts are proprietary and knowledge gained by these companies is not shared with the scientific community. Overall, there remains a lack of captive

spawning research for tropical marine fishes, despite the knowledge gaps that remain for many tropical species and the capability to do captive spawning research (Wilson et al. 2010, Curley et al. 2013, Couturier et al. 2012, Adams et al. 2014).

Bonefishes (*Albula* spp.), a tropical marine fish species, present an opportunity to test captive breeding techniques on a tropical marine fish and expand upon current knowledge of Bonefish spawning. Bonefish, along with eels, tarpon, and ladyfish, belong in the superorder Elopomorpha and research into Bonefish spawning can also provide insight into elopomorph spawning and reproductive biology, which are poorly understood.

Limited scientific knowledge exists for Bonefish (Adams et al. 2014), despite their first being described in 1758 (Linnaeus), circumtropical distribution (Adams et al. 2012), and popularity as a sportfish (Fernandez 2004). Most of the research about Bonefish has been conducted in the last three decades, coinciding with reported declines in Bonefish populations in the Florida Keys (Santos et al. 2017) and worldwide (Adams et al. 2012). Previous work on captive Bonefish has focused on their biology and stresses associated with being caught by anglers (Suski et al. 2007, Murchie et al. 2011a, Murchie et al. 2011b, Nowell et al. 2015). Captive breeding of Bonefish is a novel complement to existing scientific knowledge of these species, allowing for research insights into poorly understood early life stages as well as an important first step for a potential culture program. Successful captive spawning of eels with hormones in Japan and Europe (Ohta

et al. 1997, Sørensen et al. 2016), which are related to Bonefish, indicate that similar achievements are possible with Bonefish.

Previous work on Bonefish spawning in the field determined that spawning takes place from November to May in the Florida Keys (Crabtree et al. 1997) and around the full and new moons from October through April in The Bahamas (Danylchuk et al. 2011). Crabtree et al. (1997) also determined that Bonefish in the Florida Keys take 3 to 4 years to mature, with males maturing earlier and at smaller sizes (3.6 years, 418 mm fork length [FL]) than females (4.2 years, 488 mm FL). This information can be used to guide spawning efforts in captivity.

This study focuses on using captive breeding techniques to spawn Bonefish (*Albula* spp.) in captivity in Florida and The Bahamas. The main objectives of this chapter were induction of gonad maturation and spawning induction of captive Bonefish. Below are the results of the attempts to induce gonad maturation by environmental manipulation (i.e. photoperiod and temperature) and hormone injections and the attempts to spawn Bonefish with hormone injections.

Methods

Bonefish were collected for broodstock using hook-and-line sampling from south Florida (Biscayne Bay and the Florida Keys) and transported to facilities at Florida Atlantic University – Harbor Branch Oceanographic Institute (FAU-HBOI). Bonefish were also collected in Eleuthera, The Bahamas, using hook-and-line and seine net and transported to facilities at Cape Eleuthera Institute (CEI). Bonefish held at FAU-HBOI were placed under artificial photothermal conditions in recirculating systems to induce gonad maturation and spawning, while

Bonefish at CEI were held under ambient photothermal conditions in flow-through systems to induce gonad maturation and spawning. Hormone treatments to induce gonad maturation and spawning were administered to Bonefish at both facilities.

FAU-HBOI.

Collection.

Bonefish were collected in April and August 2016; June, July, and August 2017; and April and June 2018, from Biscayne Bay and the Middle Keys using hook-and-line (Figure 1). Captured fish were kept onboard a boat – in a flow-through live well or 300 L tank with ambient seawater and bottled oxygen – and transported to a nearby boat ramp. Fish were then transferred to an insulated 750 L or 975 L transport tank (Dyno Plastics, New Brunswick, Canada) filled with ambient seawater, and bottled oxygen was used to maintain dissolved oxygen in the tank. The tank and its lid were secured in the bed of a truck with tie-down straps for safe transport and driven to FAU-HBOI the same day. This method was used for all trips except August 2016. During the multi-day trip to the Florida Keys in August 2016, fish caught during the day were held for up to four days at the Keys Marine Lab (Layton, FL). These fish were held in 2,400 L or 4,200 L flow-through tanks and then transported back as a single group, in multiple transport tanks to avoid overstocking. All fish from the Florida Keys and Biscayne Bay were transported to FAU-HBOI (Ft. Pierce, FL, Figure 1).

Acclimation and husbandry.

At FAU-HBOI, Bonefish were quarantined at least 30 days in recirculating, 12,000 L tanks to assess general health and for prophylactic treatment of any diseases or parasites before being moved into conditioning tanks. After Bonefish successfully completed quarantine, each fish was implanted with a 12 mm HDX PIT tag (Oregon RFID, Portland, OR) in the muscle below the spiny dorsal fin for identification, measured, weighed, and cannulated to assess gonad condition, then transferred to a recirculating, 12,000 L conditioning tank. Bonefish collected in the Florida Keys were kept in a separate tank from Bonefish collected in Biscayne Bay, so that if there were any behavioral differences by region, especially relating to spawning, they were conserved. Fish were anesthetized with 86 mg/L of buffered MS-222 (Syndel USA, Ferndale, WA) in a 700 L tank during the procedure. Fin clips were also collected during transfer procedures for genetic species ID.

Water quality (dissolved oxygen, temperature, pH, and salinity) was monitored twice daily and water chemistry (alkalinity, ammonia, and nitrate) was tested twice weekly, except for two days in October 2016 and two days in September 2017 due to mandatory evacuations for Hurricane Matthew and Irma respectively. During the quarantine period and conditioning, Bonefish were fed white shrimp (*Litopenaeus setiferus*), Pacific krill (*Euphausia pacifica*), and blue crab (*Callinectes sapidus*).

Gonad maturation and spawning.

To induce gonad maturation in captivity, the tank environment was manipulated to mimic natural, annual seasonal changes in water temperature

and photoperiod (i.e. day length). Water temperature was varied between 23 °C (winter) and 29 °C (summer), matching the annual range of monthly averages observed in the Florida Keys (Crabtree et al. 1996) and photoperiod was varied between 10 (winter) and 14 (summer) hours, matching the annual cycle of photoperiod observed in the Florida Keys (Mcclintock and Stephen 1990). Water temperature was controlled with an external heat pump (Aqualogic Inc., San Diego, CA) and day length was adjusted using overhead lights and timers. Since the fish were captured during the summer, they began the conditioning cycle in summer (29 °C and 14 hour photoperiod). Fish were brought into the fall season, when gonad development and spawning is first observed in the wild, by gradually decreasing the water temperature by 1 °C/week and day length by 0.5 hr./week. Winter (i.e. spawning season) was attained once water temperature and day length reached their minimum values (23 °C and 10 hours respectively), and these values were maintained during the spawning trials (Figure 2). After 6 months, water temperatures and day length were gradually increased back to their maximum values (29 °C and 14 hours respectively) at the same rates to simulate spring and return to summer, which was used a resting period for the fish. This cycle was completed twice for the Florida Keys group and once for the Biscayne Bay group.

After the Florida Keys group finished their second full cycle, they were put onto a short cycle, which truncates spring, summer, and fall to four months instead of six months as in the full cycle, before returning to six months of winter. Temperature was changed at 1.2 °C/week and photoperiod was changed at 0.8

hr./week to complete the transition to summer in five weeks instead of six for temperature and eight for photoperiod (Figure 3). Summer (29 °C and 14 hours) was also shortened from twelve weeks to six weeks. At the end of six weeks of summer, temperature and daylength were reduced at the same rates to enter winter. Using a short cycle gets the fish out of phase with the calendar year and allowed for a third round of spawning work with the Florida Keys group to begin in June 2018 instead of waiting until October.

As Bonefish spawning is known to coincide with full moons (Danylchuk et al. 2011), moonlight was simulated to serve as a visual cue for gonad maturation and spawning. Full moons were simulated with a 50 W, 75 W, and 100 W Night Heat Lamp (Exo Terra, Mansfield, MA) during the first spawning season and Magic Light Bluetooth Pro (Schultze LLC, Scottsdale, AZ) lights for subsequent spawning seasons.

Gonad maturation was monitored when fish were under winter spawning conditions by presence or absence of gametes collected via abdominal palpating or cannulation. Fish were anesthetized with 86 mg/L of buffered MS-222 in a 700 L tank before gonadal assessment. After a fish was anesthetized, pressure was applied along the abdomen to see if any eggs or milt were released. If no gametes were released after palpating, the fish was inverted for cannulation and a plastic cannula (either 1.7 or 2.7 mm, depending on the size of the fish or resistance when inserted) was inserted into the urogenital opening until resistance was felt. Suction was applied with an attached syringe to collect gametes and the cannula was slowly removed. Samples collected from

cannulation were examined under a microscope for presence of eggs or sperm, indicating gender and gonad maturation. Egg diameters were measured under a microscope to assess spawning-readiness in females, while presence of sperm was used for assessing spawning-readiness of males. In January 2017, sonograms were taken of the Florida Keys group, using an Ibex EVO (E. I. Medical Imaging, Loveland, CO) and L14E linear transducer (E. I. Medical Imaging, Loveland, CO) set to 10 MHz, to visualize the gonads internally and determine gender, a technique that has been used successfully on other species (Karlsen and Holm 1994, Martin-Robichaud and Rommens 2001, Moghim et al. 2002, Colombo et al. 2004). For this process, the fish were anaesthetized with buffered MS-222 and placed upside-down into a v-shaped trough filled with system water, which acted as a sonic coupler for the ultrasound probe.

Bonefish were kept separated by collection region for conditioning, hormone trials, and spawning attempts. Fish that received hormone injections to promote gonad development were returned to their respective conditioning tanks (Tanks 4 or 5, Figure 4), which were also equipped with external egg collectors, in case volitional spawning occurred. For spawning attempts, after the fish received injections, they were moved from their respective conditioning tank to Systems 1 or 2 (Figure 4). Systems 1 and 2 have two, recirculating 4,500 L tanks each, equipped with skimmers and external egg collectors. The tanks (A and B) in a system are connected to the same sump and so water exchange occurs between the two tanks. Control fish were not put in the same system as fish treated with hormones to avoid any confounding effects that might result from sharing water

between tanks A and B within the same system. These tanks were used in January 2017 and 2018 for the Florida Keys group and February 2018 for the Biscayne Bay group. Fish were under daily observation for spawning behaviors or spawning after injection and the egg collectors were checked twice daily.

Hormone trials were conducted when the fish were in winter spawning season. To promote gonad development, females received an intramuscular implant of GnRHa Ovaplant (Syndel USA, Ferndale, WA), an intraperitoneal injection with carp pituitary extract (CPE, Stoller Fisheries, Spirit Lake, IA), or an intramuscular injection with human chorionic gonadotropin (HCG, Chorulon, Merck Animal Health, Madison, NJ), while the males received an intraperitoneal injection of CPE or an intramuscular injection of HCG. Hormone dosages for males and females was based on weight (e.g., 10 mg/kg). CPE and HCG were administered with 21-gauge needles and Ovaplant pellets were administered with a Ralogun (Merck Animal Health, Madison, NJ).

Cape Eleuthera Institute (CEI).

Collection.

Two groups of Bonefish were collected for spawning conditioning under ambient photothermal conditions. One group of Bonefish was collected with a seine net at the end of spawning season (April 2017) from the boathouse at CEI and the second group was collected with a seine net at the beginning of spawning season (October 2017) from Kemps Creek (Figure 5). At the boathouse, a basin (4 m deep) with one point of access to the surrounding flats, CEI divers were used to spot a school of Bonefish. Once identified, a boat was

used to deploy a 50 m seine net around the school and the CEI divers helped hold the net down while the net was brought to shore to prevent fish from escaping underneath the net. At Kemps Creek, which connects a shallow bay of intertidal flats and mangroves to coastal flats, the seine net was placed across the mouth of the creek at high tide and monitored through the falling tide, when the Bonefish were forced out of the creek and were collected in the net.

Additional Bonefish were collected by hook-and-line sampling for hormone-induced spawning experiments. Fish were collected during the spawning season in November 2017, December 2017, and January 2018 from No Name Cut (Figure 5). No Name Cut is the entrance to a series of connected basins, the deepest of which is 10 m. No Name Cut is a known pre-spawn aggregation site (PSA, Danylchuk et al. 2011), so Bonefish collected from this location were expected to have mature gonads and be spawning-capable since they made the migration to the PSA site.

All fish were transported from their respective capture locations to tanks at CEI by boat or truck. During transport, fish were held in 100 L tubs filled with ambient seawater. For longer trips, water exchanges were conducted to maintain ambient levels of dissolved oxygen and temperature.

Acclimation and husbandry.

For identification of all fish, dart tags (Hallprint, Hindmarsh Valley, South Australia) were inserted between the pterygiophores, below the dorsal fin. All fish collected were kept in systems with flow-through water, which received ambient seawater through an intake that was located 50 m off the beach at CEI. Incoming

water was not treated, but mesh bags were placed over the inflow to collect large particulates and algae before they could foul the tanks. Tanks were exposed to ambient photoperiod but were located under cover and not exposed to long periods of direct light. Fish collected from the boathouse at CEI were kept in one of four 13,000 L tanks (n=11/tank), for replication, as the long-term group (Figure 6). The long-term group, which was collected at the end of the spawning season, was held in captivity for 11 months. Bonefish collected from Kemps Creek were kept together as the short-term group in a fifth 13,000 L tank. The short-term group, which was collected at the beginning of the spawning season, was kept in captivity for four months. Bonefish collected from No Name Cut were kept in a sixth 13,000 L tank as the PSA group (Figure 6). All fish were visually monitored for health and fed daily, with a mixed assortment of shrimp, queen conch (*Lobatus gigas*) offal, or squid, by CEI staff and interns. Water temperature and conductivity were recorded with an RBR*maestro*^{3} (RBR, Ottawa, ON) every minute from 19 May 2017 to 14 January 2018 and 6 February 2018 to 08 March 2018. Fin clips were collected from the fish collected from the boathouse at CEI for genetic species ID.

Gonad maturation and spawning.

The long-term group was used to determine if wild fish collected while returning to their home range at the beginning of the rest period (i.e. not spawning) could be brought into spawning condition in captivity under ambient photothermal conditions in tanks. The short-term group was used to determine if wild fish collected from their home ranges at the beginning of the spawning

season could be brought into spawning condition in captivity under ambient photothermal conditions and induced to produce mature gametes (e.g., ovulated eggs) with hormone injections. The PSA group was used to determine if fish that had completed gonad maturation in the wild could be induced to spawn in captivity with the use of hormones.

Gonad maturation was determined by presence of gametes collected via palpating or cannulation. Fish were anesthetized with clove oil before gonadal assessment. After a fish was anesthetized, pressure was applied along the abdomen to see if any eggs or milt were released. If no gametes were released after palpating, the fish was inverted into a trough for cannulation while water was pumped over its gills.

To promote gonad development in the long-term group, fish collected from the boathouse at CEI were kept under ambient photoperiod and temperature for 11 months (April 2017 to March 2018). Fish were palpated and cannulated monthly, starting in May, to determine gender and evaluate gonad development. Presence of eggs was used to identify females, while presence of sperm was used to identify males. No hormone trials were conducted on these fish. Blood samples were collected after cannulation for a separate project. Fish were anaesthetized with clove oil (0.04 mL/L) in a 20 L tub before the procedures.

Fish from Kemps Creek were kept under ambient photoperiod and temperature for 4 months (October 2017 to January 2018) to promote gonad development and induce spawning with hormone injections during the spawning season. Sex and gonad development were checked via palpating and

cannulation before the full moon in December and January. Presence of eggs was used to identify females, while presence of sperm was used to identify males. Fish were anaesthetized with clove oil (0.04 mL/L) in a 20 L tub before cannulation.

To induce spawning in wild fish with mature gonads using hormone injections, Bonefish caught at No Name Cut were palpated and cannulated, after being brought to CEI, to assess gender and gonad development. Egg were inspected under a microscope to assess spawning-readiness in females, while presence of sperm was used for assessing spawning-readiness of males. Fish of undetermined gender were held for up to two months for observation of gonad development. Gravid females received injections of CPE (10, 20, or 30 mg/kg) to promote oocyte growth and DHP (1 or 2 mg/kg) to induce final oocyte maturation and ovulation. Males with sperm received HCG (1,000 IU/kg) to induce sperm production. After injections, fish were returned to holding tanks or moved to 1,600 L, tanks when available, for easier access during observations. Fish were anaesthetized with clove oil (0.04 mL/L) in a 20 L tub before cannulation and injections.

Results

FAU-HBOI.

Collection.

Florida Keys.

Bonefish collected from the Florida Keys (n=23) ranged from 324 to 530 mm FL (n=21, mean=408 ± 50 mm) and weights ranged from 586 to 2,640 g

(n=23, mean=1,247 ± 482 g, Table 1). No mortalities occurred during capture or transport.

Biscayne Bay.

Bonefish were collected from Biscayne Bay (n=23) ranged from 405 to 630 mm FL (n=23, mean=474 ± 54 mm) and weights ranged from 955 to 4,340 g (n=23, mean=1,828 ± 724 g, Table 2). No mortalities occurred during capture or transport.

Acclimation and husbandry.

Florida Keys group.

All fish were held for 30 days in quarantine. One mortality occurred in quarantine shortly after transfer and was determined to be due to handling stress from capture and transport. Another mortality occurred in quarantine during Hurricane Matthew after a fish jumped through a small gap in the net covering the tank. The remaining fish (n=21) from this group successfully completed quarantine, showing no signs of disease or parasites.

After completing quarantine, all fish were transferred to System 4 (Figure 4). Fish were moved between System 4 and System 5 after gonad assessment or spawning trials until September 2017, when the Biscayne group was placed in System 4 and the Keys group remained in System 5. Water temperature for the Florida Keys group ranged from 20.6 °C (winter) to 29.6 °C (summer) in accordance with the conditioning cycle phase (Figure 2, Figure 3). Average salinity was 30.8 ± 2.1 ppt (n=603) and ranged from 25.5 ppt to 35.3 ppt. The variation in salinity was due to the refilling of the tanks with lower salinity source

water (ranging from 25-28 ppt) after gonad assessment, which was gradually increased back to the maintenance salinity of 30 ppt, and an attempt to vary salinity seasonally at the beginning of the second summer, which was discontinued after a series of unrelated mortalities. The remaining tank parameters were not varied seasonally. Average dissolved oxygen (DO) was 126 ± 9 % saturation (n=1,205); average pH was 8.02 ± 0.20 (n=602); average alkalinity was 228 ± 44 mg/L (n=167); average total ammonium nitrate (TAN) was 0.05 ± 0.04 mg/L (n=164); and average nitrite was 0.099 ± 0.092 mg/L (n=163). Variation in TAN and nitrite was due to changes in rations. Fish were fed to satiation daily (0 to 2,050 g/tank).

Microsatellite testing of the fin clips collected when the fish were transferred out of quarantine (n=20) determined that fifteen fin clips were collected from *Albula vulpes* (Table 1). The other five fin clips provided insufficient genetic material for species identification and fin clips taken from the three fish captured after the initial species ID were not submitted for identification.

Bonefish that had been in captivity for over a year were remeasured in December 2017 for growth and so that hormone dosages could be adjusted by weight (Table 1). Lengths ranged from 395 to 530 mm (n=15, mean=480 ± 40 mm) and weights ranged from 1,180 to 2,640 g (n=15, mean=2,079 ± 515 g), which were greater than the measurements taken in 2016.

Biscayne group.

All fish were held for 30 days in quarantine. No mortalities were observed and all fish from this group successfully completed quarantine, showing no signs of disease or parasites. After completing quarantine, all fish were transferred to System 4 (Figure 4). Water temperature ranged from 23.5 °C (winter) to 30.2 °C (summer) in accordance with the conditioning cycle phase (Figure 3). The remaining tank parameters were not varied seasonally. Average salinity was 29.8 ± 2.4 ppt (n=271); average DO was 123 ± 8 % saturation (n=541); average pH was 8.08 ± 0.13 (n=271); average alkalinity was 223 ± 56 mg/L (n=75); average TAN was 0.05 ± 0.06 mg/L (n=75); and average nitrite was 0.065 ± 0.075 mg/L (n=75). Variation in TAN and nitrite was due to changes in rations. Fish were fed to satiation daily (0 to 2,150 g/tank).

Microsatellite testing of the fin clips collected when the fish were transferred from quarantine (n=2) determined that both fin clips were collected from *Albula vulpes* (Table 2). Fin clips were collected from the other fish captured in Biscayne Bay (n=21) after the initial species ID and were not submitted for identification.

Gonad maturation and spawning.

Florida Keys group.

Bonefish in the Florida Keys are reproductively active from November to May (Crabtree et al. 1997), so gender and reproductive status were first assessed on 10 November 2016, four days before the full moon. Two fish (502 and 517) were confirmed as females due to eggs collected via cannulation

(Figure 8). Measurements were not taken, but visual inspection of the eggs under a microscope indicated that the eggs were not well developed based on color and opacity. The remainder (n=18) were of undetermined gender. No hormones were administered and no spawn was observed (Table 3).

One fish (572) died on 8 January 2017. It had previously jumped through a small gap in the net a few days prior and been returned to the tank. The cause of death was determined to be injuries sustained from and stress associated with jumping out of the tank. On 25 January 2017, two days before the new moon, Bonefish gender and reproductive status were assessed using cannulation and ultrasound (n=19). Sperm was collected from one male (596) after it was palpated (Figure 8, Table 4). Using the ultrasound, sonograms of the ovaries of the two known females (502 and 517, Figure 7) were taken and used as a reference to identify six more putative females (510, 512, 529, 548, 593, and 518). Using the male as a reference, seven more putative males (567, 514, 575, 538, 561, 504, and 532) were identified by sonograms of the testes (Figure 7).

Hormone injections were administered to four females and six males to conduct a spawning trial. A 75 mg Ovaplant pellet was injected into the musculature of two females (502 and 517). The other two females (593 and 529) received a sham injection (i.e. no hormone was administered) as a control. Six males (567, 596, 538, 532, 561, and 504) received a 1,000 IU injection of HCG (Table 4). After injections, the fish were moved to System 1 and 2 (Figure 4) for observation. Tanks 1A and 1B had one female and two males. Tanks 2A and 2B had one female and one male. No spawn was observed in any tank after 120

hours. On 30 January 2017, fish were recaptured and inspected. No eggs or milt were cannulated, strip spawning was unsuccessful, and the fish were returned to the conditioning tank.

No further attempts were made for that spawning season and water temperature and photoperiod were increased, transitioning the fish into the summer resting period. Blood samples were taken in May 2017 for a separate project. Between June and August 2017, seven fish died (502, 567, 512, 561, 596, 543, and 577). Mortalities occurred one at a time and several days apart. Necropsies and pathology by Fishhead Labs LLC (Stuart, FL) concluded the cause of death was brain hemorrhaging, likely sustained from swimming into the sides of the tank at high speeds when startled. Three fish (079, 845, and 035) were added to the tank in September 2017.

On 4 November 2017, the day of the full moon, gender and reproductive status were assessed (n=15). No gametes were collected during cannulation, no hormones were administered, and no spawn was observed (Table 5).

On 4 December 2017, the day after the full moon, gonad assessment was conducted (n=15) and no gametes were collected from cannulation. To induce gonad maturation, three females (529, 548, and 593) received CPE injections (10 mg/kg) and one female (517) received an injection of HCG (1,000 IU). Four males (575, 538, 504, and 532) and one undetermined fish (079) received 1,000 IU injections of HCG. Fish were returned to the conditioning tank for observation. No spawn was observed (Table 6).

On 31 December 2017, the day before the full moon, gender and reproductive status were assessed (n=15). Eggs were collected from one female (079) and the fish received an injection of CPE (10 mg/kg) before being returned to the conditioning tank. No gametes were collected from the other fish and no other injections were administered. No spawn was observed (Table 7).

On 31 January 2018, the day of the full moon, gender and reproductive status were assessed (n=15). One female fish (079) had eggs when cannulated (Figure 8). The eggs were between 200-300 microns in diameter. No gametes were collected from the other fish. To induce gonad maturation, each fish (n=15) received a CPE injection. Fish 079 received 20 mg/kg and the remainder received 10 mg/kg. Fish 079 was placed in a System 1A for observation and the remaining fish were returned to the conditioning tank. On 2 February 2018, fish 079 was cannulated to check egg development and the eggs were between 320-375 microns. The fish was returned to Tank 1A. On 4 February 2018, fish 079 was checked again for egg development and the eggs were 300 microns. A second dose of CPE was administered (20 mg/kg) and the fish was returned to Tank 1A. On 6 February 2018, fish 079 was checked for egg development and the eggs were 330 microns. No spawn was observed (Table 8). The fish was returned to the conditioning tank.

On 28 February 2018, the day before the full moon, gender and reproductive status were assessed (n=15). Sperm was collected from one male (538) and eggs, which were 200 microns, were collected from one female (079, Figure 8). Six females (510, 529, 517, 548, 593, and 097), one male (514), and

three undetermined fish (523, 845, and 035) received 20 mg injections of CPE. Four males (575, 538, 504, and 532) and one female (518) received 1,000 IU injections of HCG. All fish were returned to the conditioning tank and no spawn was observed (Table 9).

No further hormone trials were conducted and the fish were put into a short cycle (i.e. shorter summer, Figure 3), so that they would be back in winter conditions by June instead of October. During the short cycle, two fish died (504 and 523) and one fish (942) was added to the group. Cause of mortality was determined to be brain hemorrhaging for both fish.

On 7 June 2018, eight days after the full moon, gender and reproductive status were assessed (n=14). Eggs were collected from two females (518 and 079) and no gametes were collected from the other fish (Figure 8). Eggs from fish 518 were 100 microns and eggs from fish 079 were 300-375 microns. One male (538), one female (518), and one undetermined fish (035) received 1,500 IU injections of HCG. One female (593) received a sham injection. The remaining fish received different dosages of CPE: two females (548 and 517) and one undetermined fish (942) received 10 mg/kg; two females (079 and 510) and two males (575 and 532) received 20 mg/kg; and one female (529), one male (514), and one undetermined fish (845) received 30 mg/kg. All fish were returned to the conditioning tank. On 8 June 2018, a new fish (956) was moved from quarantine to the conditioning tank and received a 25 mg/kg injection of CPE. Gender or gonad maturation was not assessed for this fish. No spawn was observed (Table 10).

On June 26th, one fish died (517), likely due to handling stress during the previous hormone manipulation. On June 28th, the day after the full moon, fish were checked for gender and reproductive status (n=14). Two fish had eggs when cannulated (518 and 079) and no gametes were collected from the other fish (Figure 8). Eggs from fish 518 were 100 microns and eggs from fish 079 were 300-375 microns. One male (538), one female (518), and one undetermined fish (035) received 1,500 IU injections of HCG. One female (593) received a sham injection. The remaining fish received different dosages of CPE: one female (548) and one undetermined fish (942) received 10 mg/kg; two females (079 and 510) and two males (575 and 532) received 20 mg/kg; one undetermined fish (956) received 25 mg/kg; and one female (529), one male (514), and one undetermined fish (845) received 30 mg/kg. All fish were returned to the conditioning tank. No spawn was observed (Table 11).

On July 7th, one fish died (518) and another died on July 24th (575), both likely due to handling stress during the previous hormone manipulation. On July 27th, the day of the full moon, fish were checked for gender and reproductive status (n=12). No fish had gametes when cannulated. One male (538) and one undetermined fish (035) received 1,500 IU injections of HCG. One female (593) received a sham injection. The remaining fish received different dosages of CPE: one female (548) and one undetermined fish (942) received 10 mg/kg; two females (079 and 510) and one male (532) received 20 mg/kg; one undetermined fish (956) received 25 mg/kg; and one female (529), one male

(514), and one undetermined fish (845) received 30 mg/kg. All fish were returned to the conditioning tank. No spawn was observed (Table 12).

Of the 23 Bonefish in the Florida Keys group, two females and one male (13%) developed gametes capable of being collected via cannulation due to environmental manipulation. Two females developed eggs after receiving injections of HCG and one male developed sperm after receiving an injection of CPE (15%). The remaining 17 fish did not develop gametes as a result of environmental manipulation or hormone injections (Figure 8).

Biscayne group.

The Biscayne Bay group was on a natural conditioning cycle, but because fish were added to the tank later (September), the fish were not assessed until later in the spawning season to allow time for the new fish to acclimate and condition. Gender and reproductive status were assessed (n=20) on 1 February 2018, the day after the full moon. Six males (995, 522, 918, 904, 922, and 982) were identified by sperm collected from cannulation (Figure 9). None of the males were running (i.e. sperm expressed after light abdominal pressure). Sperm motility was confirmed using a microscope. Four females (928, 970, 980, and 999) were identified by eggs collected from cannulation (Figure 9). Fish 928 had 300 micron eggs; fish 970 had 500-600 micron eggs; fish 980 had 250-300 micron eggs; and fish 999 had 100 micron eggs. The remaining ten fish were undetermined. No fish was ready to spawn. Two females (928 and 970) received 25 mg/kg injections of CPE. The other two females (980 and 999), two males (995 and 982), and six undetermined fish (929, 954, 930, 957, 924, and 886)

received 10 mg injections of CPE. Four males (522, 918, 904, and 922) and four undetermined fish (983, 541, 962, and 938) received 1,000 IU injections of HCG. Two females (928 and 970) and one male (904) were moved into System 1B for a spawning trial. The remaining fish were returned to the conditioning tank. On 2 February 2018, the two females were cannulated to check egg development. Fish 928 had 300 micron eggs; fish 970 had 600 micron eggs. The fish were returned to the spawning tank. The male was not checked. On 4 February 2018, the two females were cannulated to check egg development. Fish 928 had 300 micron eggs; cannulation was unsuccessful for fish 970. A second dose of CPE was administered. Fish 928 received 25 mg/kg and fish 970 received 30 mg/kg. Both fish were returned to the tank. The male was not checked. On 6 February 2018, the females were cannulated to check egg development. Fish 928 had 300 micron eggs; fish 970 had 650-750 micron eggs. The male was not checked. All three fish were returned to the conditioning tank. No spawn was observed in the spawning or conditioning tank (Table 13).

On 1 March 2018, the day of the full moon, gender and reproductive status were assessed (n=20). Sperm was cannulated from two males (983 and 938) and no gametes were collected from the other fish (Figure 9). The four females (928, 970, 980, and 999) and one undetermined fish (929) received CPE injections. Fish 929 received 15 mg; fish 928 received 20 mg; fish 970 received 60 mg; fish 980 received 20 mg; and fish 999 received 15 mg. Eight males (995, 522, 918, 983, 904, 922, 938, and 982), three undetermined fish (541, 962, and 930) received 1,500 IU injections of HCG. The remaining four undetermined fish

received no injections. All fish were returned to the conditioning tank and no spawn was observed (Table 14). No further assessments or injections were done on the fish.

Of the 23 Bonefish from the Biscayne group, four females and six males (43%) developed gametes capable of being collected via cannulation due to environmental manipulation. Two males developed sperm after receiving injections of HCG (15%). The remaining 11 fish did not develop gametes as a result of environmental manipulation or hormone injections.

CEI.

Collection.

Long-term group.

Bonefish were collected with a seine net from the boathouse of CEI in April 2017 (n=44, Table 17, Table 18). Fish ranged in length from 294 to 460 mm FL (n=36, mean=352 ± 32 mm) and weight from 282 g to 1,041 g (n=31, mean=517 ± 148 g). No mortalities occurred during capture or transport. Fish were dart tagged and twelve fish had to be retagged when their original tags fell out. Dart tags fell off one at a time, so it was possible to identify the previous tag number by process of elimination and connect the new and old tag numbers in record keeping.

Short-term group.

Bonefish were collected with a seine net from Kemps Creek in October 2017 (n=31). Fish were dart tagged for identification but no lengths or weights

were taken to minimize handling stress. No mortalities occurred during capture or transport.

Pre-spawn aggregation group.

Bonefish were collected with hook-and-line from No Name Cut, a known Bonefish PSA site, in early December 2017 (n=15, Table 19), late December 2017 (n=10, Table 20), and late January 2018 (n=10, Table 21). Lengths ranged from 308 to 452 mm FL (n=28, mean=364 ± 35 mm) and weights ranged from 413 to 1,012 g (n=7, mean=664 ± 221 g). Fish were dart tagged, except for five fish in January because they were only being held for a few days in a separate tank and were running males or undetermined. No mortalities occurred during capture or transport.

Acclimation and husbandry.

Long-term group.

Fish were placed into one of four conditioning tanks (n=9) after capture (Figure 6). Bruising and discoloration were observed on the Bonefish from capture with the seine net (n=36). Both symptoms abated after one week, no infections manifested, and no mortalities were observed. Eight fish caught later in April were distributed evenly among the tanks. Tanks were outside under a pavilion (roof but no walls), under ambient light regime, and on flow-through water supply. Water temperature and salinity were recorded from 19 May 2017 to 14 January 2018 and 6 February 2018 to 8 March 2018. All fish were released in March 2018. Average water temperature was 27.92 ± 2.66 °C (n=354,234) and ranged between 17.50 °C (January) and 32.75 °C (August). Average salinity was

32.97 ± 4.11 ppt and ranged between 22.13 ppt (December) and 39.79 ppt (June). One fish died on 13 November 2017 and another died 02 March 2018. Causes of death were undetermined.

Microsatellite testing of the fin clips collected when the fish were first evaluated in April (n=35) determined that from the 26 fin clips were collected, 17 were *Albula vulpes*, eight were *Albula goreensis* and one was a hybrid between the two species (Table 17, Table 18).

Short-term group.

Fish were placed into their own conditioning tank (Figure 6). Bruising and discoloration were observed on the Bonefish from capture with the seine net. Both symptoms abated after a week, no infections manifested, and no mortalities were observed. The tank was under a pavilion, under ambient light regime, and on flow-through water supply. Water temperature and salinity were recorded from 19 October 2017 to 14 January 2018 and 6 February 2018 to 8 March 2018. All fish were released in March 2018. Average water temperature was 25.16 ± 1.98 °C (n=134,541) and ranged between 17.50 °C (January) and 29.39 °C (October). Average salinity was 28.51 ± 2.41 ppt (n=134,143) and ranged between 22.13 ppt (December) and 35.63 ppt (February).

Pre-spawn aggregation group.

Fish were transported from the PSA back to CEI (Figure 5) with no mortalities. All fish collected from the PSA site were placed into a separate tank from the other two groups (Figure 6). The tank was under a pavilion, under ambient light regime, and on flow-through water supply. Water temperature and

salinity were recorded from 2 December 2017 to 14 January 2018 and 6 February 2018 to 8 March 2018. All fish were released in March 2018. Average water temperature was 23.90 ± 1.73 °C (n=71,181) and ranged between 17.50 °C (January) and 26.98 °C (March). Average salinity was 28.16 ± 2.78 ppt (n=71,181) and ranged between 22.13 ppt (December) and 35.63 ppt (February).

Gonad maturation and spawning.

Long-term group.

To assess gonad maturity and gender, fish from each tank were cannulated monthly from May 2017 to March 2018 (n=10) as well as a final check on all fish in March before they were released. Overall, four females and six males were identified based on gametes collected during cannulation (Figure 10). In Tank 1, eggs were collected four times (November, December, February, and March) from one female (HOP6897) and sperm was collected once (March) from one male (HOP6355). In Tank 2, sperm was collected from two males in December (HOP6894) and March (HOP6894 and HOP6381). In Tank 3, eggs were collected one time (February) from a female (HOP6379) and sperm was collected one time (March) from one male (HOP6376). In Tank 4, eggs were collected in January (HOP6358 and HOP6371), February (HOP6358), and March (HOP6358 and HOP6371) from two females and sperm was collected once (March) from two males (HOP6891 and HOP5533). No spawning behavior or spawning was observed in any of the tanks.

In the long-term group, ten fish (23%) produced gametes. Four females developed eggs and six males produced sperm in response to changes in

photothermal conditions. However, no spawn was observed in any of the tanks. No gametes developed from June through October, which is consistent with previous research on bonefish maturity and spawning in The Bahamas (Danylchuk et al. 2011). The first gametes collected were from Tank 1 in November (Figure 10) and of the ten fish that produced gametes between then and March nine had gametes in March.

Short-term group.

To assess gonad maturity and gender, fish were cannulated before the full moon in December 2017 and January 2018. Eleven fish were cannulated in December and no gametes were collected (Table 22). The remainder were left alone until the following full moon to minimize handling stress. A total of 31 fish were cannulated in January and no gametes were collected (Table 22). Without positive identification of gonad maturation and at least one female and male, no hormone injections were administered and the fish were released.

Pre-spawning aggregation group.

To assess gonad maturity and gender, fish collected from No Name Cut were transported to CEI, where they were palpated or cannulated within 24 hours. Overall, seven females and sixteen males were identified by gametes collected from cannulation or, for some males, the expression of milt when palpated. Of the fifteen fish collected in early December (Table 19), four females had eggs when cannulated (HOP6825, HOP6826, HOP6830, and HOP6888), three males expressed milt when palpated (HOP6895, HOP6893, and HOP6882), and three males had sperm when cannulated (HOP6824, HOP6828,

and HOP6829). Of the eleven fish collected in late December (Table 20), three females had eggs when cannulated (HOP6914, HOP6915, and HOP6916) and one male was running ripe (HOP6913). Of the ten fish caught during late January (Table 21), five were running males (HOP6901, HOP6902, HOP6903, HOP6904, and HOP6905) and two males had sperm when cannulated (not tagged).

To induce spawning in the fish from December, seven of the ten Bonefish with mature gonads received hormone injections. HOP6825 and HOP6826 (females) received an injection of CPE (10 mg/kg) on December 4th to stimulate oocyte maturation (Table 23). On December 5th, 23 hours after the CPE injection, both fish received an injection of DHP (2 mg/kg) to induce ovulation and spawning. Spawning did not occur within 24 hours, so a second injection of DHP (2 mg/kg) was administered on December 6th. No spawn was observed within 24 hours from either fish and no more hormones were administered. HOP6830 (female) received an injection of CPE (10 mg/kg) on December 4th to stimulate oocyte maturation. On December 5th, 23 hours after the CPE injection, HOP6830 received an injection of DHP (4 mg/kg) to induce ovulation and spawning. Spawning did not occur within 24 hours, so a second injection of CPE (10 mg/kg) was administered to continue oocyte maturation and induce spawning. No spawn was observed within 24 hours and no more hormones were administered. HOP6888 (female) received an injection of CPE (10 mg/kg) on December 5th to stimulate oocyte maturation. No injections were administered on December 6th to allow more time for oocyte maturation to occur without handling. On December 7th, HOP6888 was injected with DHP (2 mg/kg) to induce ovulation and

spawning. Spawning did not occur within 48 hours and no more hormones were administered. Three males (HOP6824, HOP6828, and HOP6829) received injections of HCG (2,000 IU/kg for HOP6824 and HOP6828; 1,000 IU/kg for HOP6829) on December 4th to stimulate sperm production. HOP6824 was running ripe on December 5th, but neither of the other males were. The other three males (HOP6882, HOP6893, and HOP6895), which were already running ripe, did not receive any injections. Two males (HOP6824 and HOP6895) died a few days after the spawning trial. Cause of death was determined to be from handling and associated stress.

To induce spawning in the fish from late December, two females (HOP6915 and HOP6916) were injected with hormones (Table 24). HOP6915 received an injection of CPE (20 mg) on December 31st to stimulate oocyte maturation. On January 1st, HOP6915 received another injection of CPE (20 mg) to continue stimulating oocyte maturation and induce spawning. Eggs had a diameter of 1,220 µm (n=7). On January 2nd, while being anaesthetized for a third injection of CPE, HOP6915 began spontaneously releasing eggs. The fish was immediately strip spawned into a separate container and the eggs were fertilized with sperm from the running male (HOP6913). Eggs were incubated and hatched and the larvae lived for 56 hours, by which point all had died.

HOP6916 received an injection of CPE (10 mg) on December 30th to stimulate oocyte maturation. Egg diameters were 675 µm (n=12). On December 31st, HOP6916 received a second injection of CPE (10 mg) to continue stimulating oocyte maturation and induce spawning. Egg diameters were 971 µm

(n=12). On January 1st, 2 mg of DHP was injected into the fish to induce ovulation and spawning. The next day, the fish had decreased notably in girth, similar to HOP6915, and likely spawned in the tank overnight. No egg collectors were present on the tank and the eggs would have been flushed out of the bottom the tank before being collected. No hormones were administered to the male fish (HOP6913), as it was already running ripe.

Because no females were identified from the fish captured at the PSA in late January (Table 21), previously identified females from December and January received hormone injections to induce gonad maturation and spawning (Table 25). One female from December (HOP6830) and two females from January (HOP6914 and HOP6915) as well as one male from January (HOP6913) were injected. Only HOP6914 had eggs, which measured 700 µm. On January 29th, all three females received an injection of CPE (10 mg) and the male received an injection of HCG (1,000 IU). Several running males were later captured at the PSA and HOP6913 received no further hormone injections. No injections were administered on January 30th. On January 31st, HOP6915 and HOP6830 received 10 mg injections of CPE and HOP6914, whose eggs had increased to1,000 µm, received a 30 mg injection of CPE. No eggs were collected during cannulation from HOP6915 or HOP6930 on January 31st or February 1st and hormone injections ceased. HOP6914 received another injection of CPE (30 mg) on February 1st and 2nd and egg diameters did not change. Ultimately, on February 3rd, HOP6914 died in the tank. A necropsy was performed and the ovaries were fully developed and filled the coelomic cavity. No

spawn was observed and no attempt was made to fertilize the eggs found in HOP6914 during the necropsy.

The genders of 21 fish (60%, seven females and fourteen males) were identified over three months. Four females and three males received hormone injections in early December (Table 19), but the hormones did not induce spawning (Table 23). Two females received hormone injections in late December which resulted in one confirmed spawn from one female, that was fertilized by a male that was already running, and one likely, but unconfirmed, spawn from the other female (Table 24). Three females from the previous two collections and one male collect in late January received hormone injections (Table 25), and while the hormones did not induce spawning, advanced ovarian development was observed during necropsy of the one female that died.

Discussion

Photothermal manipulations have been used successfully to spawn several species in captivity, however these species tend to be temperate (Wang et al. 2010), where the magnitude of shifts in daylength and water temperature are higher than in the tropics (Ebeling and Hixon 1991). Bonefish, which are a tropical species, were reared under ambient and artificial photothermal conditions in this study and neither were as effective at promoting gonad development or spawning as in temperate fish. Gonad development occurred in some fish, but many showed no signs of development, and no spawning occurred. In the tropics, where average annual rainfall is higher than temperate regions (Boyer et al.1999), perhaps fluctuations in salinity represent a third environmental cue that

some tropical fish may also need to synchronize gonad maturation and spawning. Osmoregulating has impacts on the endocrine functions of marine teleosts (McCormick 2001) and changes in salinity may serve as a cue or allow for proper reproductive hormone action. Salinity in Florida Bay fluctuates up to 10 ppt intra-annually in response to freshwater flow from the Everglades (Boyer et al. 1999) and decadal hypersalinity events in Florida Bay lead to even larger variability in salinity (Fourqueran and Robblee 1999). Similar variation is also seen in Mexico (Morales-Vela et al. 2000, Vásquez-Yeomans 2009) at locations where Bonefish occur (Adams et al. 2007, Vásquez-Yeomans 2009). At Cape Eleuthera during this study, a difference of 17 ppt was observed between the summer and winter, although it is unclear if this is a natural phenomenon or anthropogenically driven. Larger fluctuations (18-35 ppt) were observed at the cays and atolls of Belize (Platt et al. 2013), where Bonefish also reside (Bates 2017)

Likewise, hormone injections have been used successfully to induce gonad maturation and spawning in multiple species (Zohar and Mylonas 2001, Duncan et al. 2003, Hill et al. 2009, Miller 2009). The hormones used in the study are used effectively to induce gonad maturation in European Eel (Palstra et al. 2005, Mordenti et al. 2012) and other pituitary extracts have been used successfully in other eel species (Ohta et al. 1996b, Lokman and Young 2000). Given the close lineage of Bonefish to eels within the Superorder Elopomorpha, it was assumed that hormones used successfully in eels would also be effective in Bonefish. Ovaplant, which is not used for eels, is used successfully in other

species such as Florida Pompano (*Trachinotus carolinus*), Atlantic Croaker (*Micropogonias undulatus*) and Golden Trevally (*Gnathanodon speciosus*) (Weirich and Riley 2007, Sink et al. 2010, Broach et al. 2015). As this study was the first to use hormone injections on Bonefish, their sensitivity to the hormones used was unknown. However, it seems unlikely that none of the hormones currently in use for captive research in fish would be effective for Bonefish.

Captivity results in some level of reproductive dysfunction for all fish, due to inadequacies in replicating spawning conditions and cues, stress from being in a confined space, changes in behavior, and the presence of humans (Zohar and Mylonas 2001, Mylonas and Zohar 2007). This dysfunction associated with captivity may have inhibited Bonefish gonad maturation, despite receiving appropriate photothermal cues. Aside from the stress associated with captivity, all fish were handled multiple times for capture, gonad assessment, or hormone injections. Elevated levels of cortisol were observed in *A. glossodonta* and *A. vulpes* after exertion or capture (Friedlander et al. 2007, Shultz et al. 2011). Elevated levels of cortisol have been shown to disrupt reproductive hormone levels, gonad maturation, and spawning in several species (Barton and Iwama 1991, Pankhurst and Van Der Kraak 1997) and repeated handling can cause mortality (Zohar and Mylonas 2001). Additionally, previous studies on Bonefish handling stress associated with angling events reported that exposure of the gills to air is stressful for Bonefish (Suski et al. 2007, Cooke et al. 2008), so in this study Bonefish were exposed to air briefly and infrequently during procedures to minimize associated stress. To counteract the effects of handling stress,

anesthesia was used to sedate Bonefish and minimize stress before being assessed or injected. The effects of MS-222 and clove oil in reducing stress response in Rainbow Trout (*Oncorhyncus mykiss*) were similar (Wagner et al. 2003), so it is assumed that the use of MS-222 at FAU-HBOI and clove oil at CEI did not mitigate stress better for one group.

Another possible explanation for why photothermal manipulation and hormone injections were ineffective is immaturity. Work done by Crabtree et al. (1997) on Bonefish maturity in the Florida Keys found that 50% maturity was reach at 418 mm FL for males and 488 mm FL for females. Several of the fish collected from the Florida Keys and Biscayne Bay were below these lengths and thus less likely to be mature. No study to date has established a length-maturity relationship for Bonefish in The Bahamas, but in other locations in the Greater Caribbean maturation occurs at shorter lengths (Adams et al. 2007) than in the Florida Keys. So, the smaller sizes observed at CEI relative to the fish at FAU-HBOI does not necessarily mean they were immature.

Proper diet is important for somatic and gonadal growth when rearing fish in captivity. Diets with insufficient calories or lacking in essential amino acids can prevent gonad development or result in inferior gonads and gamete quality (Izquierdo et al. 2001). Bonefish at FAU-HBOI were fed diets that closely resembled diets of wild Bonefish (Crabtree et al. 1998) and fish at CEI were fed a diet that has been used in previous Bonefish studies at that location (Murchie et al. 2009, Murchie et al. 2011, Stein et al. 2012).

Each of these factors likely had some impact on gonad maturation and spawning for the Bonefish in this study. Below is a more detailed look at how each of the factors may have impacted the maturation and spawning work for the Bonefish at FAU-HBOI and CEI.

FAU-HBOI.

Photothermal conditions for the Bonefish at FAU-HBOI were artificial simulations of annual variations in daylength and water temperature reported in the Florida Keys (Crabtree et al. 1996, Mcclintock and Stephen 1990) and lunar cycles (i.e. occurrence of the full moon). This resulted in 13% of the Florida Keys group and 43% of the Biscayne Bay group developing gametes, but not completing maturation, during the natural conditioning cycle (Figure 2). The difference in effectiveness between groups on the natural cycle is unusual, as photothermal conditions were maintained at the same level. Efforts were made to collect fish from the Florida Keys and Biscayne Bay that were larger than 406 mm FL so that they were likely sexually mature (Crabtree et al. 1997), but some of the fish were shorter and may have been immature (Table 1, Table 2). Immaturity may have affected the fish in the Florida Keys group during the first conditioning cycle, when they were smaller (mean=408 FL). However, in the second conditioning cycle, the fish were larger (mean=480 FL) and should have been large enough to have reached maturity (Crabtree et al. 1997). Despite their increase in length and weight (Table 1), eggs were collected from only two fish and sperm was collected from one. It is unclear if being in captivity may have inhibited the transition from immaturity to maturity. Both groups appeared to have

the same amount of stress during handling, but no additional analyses were done to verify if that was the case. After the Florida Keys group were put onto a short cycle (Figure 3), 13% of the fish developed gametes. Since this was the same as on the natural cycle and did not appear to disrupt gonad maturation, short-cycling may be a useful technique for accelerating future captive research with Bonefish

Salinity variation as a missing cue for gonad development was considered after the first natural conditioning cycle for the Florida Keys group. During the summer of second cycle for the Florida Keys group, salinity was increased by 1 ppt/week until reaching 35 ppt, which salinities in Florida Bay meet or exceed during the summer (Kelble et al. 2007), but this experiment was terminated after several mortalities occurred in the tank, which were later attributed to brain hemorrhaging and likely not related to the salinity manipulations. Since it was during the summer, no fish were checked for gonad development, although none would have been expected.

Hormone injections of CPE and HCG were ineffective at inducing gonad maturation (Table 15, Table 16). Some of the fish that did respond to hormone injections had the opposite reactions than expected. Two females (518 and 079) developed eggs one month after HCG injections, which is primarily used to induce sperm production (Ohta et al. 1996a, Lokman and Young 2000), and one male developed sperm one month after receiving an injection of CPE, which is primarily used for oocyte production and growth (Palstra et al. 2005, Mordenti et al. 2012). However, HCG has been used for gonad development and spawning for female flatfish, Pinfish (*Lagodon rhomboides*), and Pigfish (*Orthopristis*

chrysoptera) (Canario and Scott 1990, DiMaggio et al. 2013, DiMaggio et al. 2014) while CPE, paired with GnRH, has been used to induce spermiation in European Catfish (*Silurus glanis*) (Linhart and Billard 1994).

None of the three attempts to spawn Bonefish at FAU-HBOI using hormone injections were successful. This is likely due to the lack of females with eggs mature enough that the hormones would induce spawning as opposed to the inefficacy of hormone injections to induce spawning. Bruger (1974) reported eggs as large as 700 µm for the vitellogenic stage, but did not have any eggs from the maturation stage, which presumably are larger. The eggs of Japanese Eels (*Anguilla japonica*) in captivity are only considered mature when they reach sizes greater than 700 µm (Kagawa et al. 1995). Only one fish (970) had eggs large enough (500-600 µm) to be close to the maturation stage, while the other eggs were smaller and either early vitellogenic or previtellogenic (Bruger 1974).

Sonograms from the Florida Keys group provided putative genders for 16 fish, but only two fish were later confirmed based on gametes collected and one fish from necropsy. Another fish, identified as a male with the ultrasound, had ovaries during necropsy. While the use of ultrasound for other fish species has successfully identified gender (Karlsen and Holm 1994, Martin-Robichaud and Rommens 2001, Moghim et al. 2002, Colombo et al. 2004), in this study, genders determined from sonograms may have been inaccurate.

The diet for Bonefish at FAU-HBOI was comprised of shrimp and blue crab, both of which Bonefish are known to eat in the wild (Crabtree et al. 1998), and krill. While krill is not a natural food item for Bonefish, it was included for

amino and fatty acid supplementation (Izquierdo et al. 2001) and the Bonefish consumed it. In larger Bonefish in the Florida Keys, toadfish (*Opsanus beta*) become a prey item (Crabtree et al. 1998). Attempts were made to incorporate silversides (*Menidia* spp.) into the diet, as a substitute for a toadfish, but the Bonefish would not eat them and seemed to actively avoid them. It is possible that the lack of fish, essential nutrients, or fatty acids in the diet was negatively impacting gonad maturation (Izquierdo et al. 2001). While Bonefish in both groups did not eat for up to a week after handling (i.e. movement between tanks, gonad assessment), when they resumed eating they were fed to satiation, which was as much as 6% of body weight for both tanks. This is higher than optimal feed rates of 1% for Nile Tilapia (*Oreochromis niloticus*) (Tsadik and Bart 2007) but lower than feed rates of 10% used for Redfish (*Sciaenops ocellatus*) (C. Robinson, personal communication). The fish that were in captivity for over a year in the Florida Keys group increased in both lengths and weights (Table 1) at rates similar to those observed in the Florida Keys (Crabtree et al. 1996), indicating they were fed enough for somatic growth. The abundant fat stores found during necropsies were also evidence that they were receiving enough food during the study. However, this may also be an indication that the fish were overfed and this excess may have also impacted gonad development (Izquierdo et al. 2001).

Based on all of the above, the following suggestions are made to improve the rate of gonad maturation and spawning in future induction studies with Bonefish from the Florida Keys and Biscayne Bay. To determine if salinity is an

environmental factor affecting gonad development in Bonefish, long-term salinity measurements should be taken from Bonefish home range and pre-spawn habitats and compared to the reproductive status of Bonefish captured from those locations. Handling stress can be reduced by replicating this study and examining the fish less frequently or stocking fish in a tank, leaving them alone, and watch for any volitional spawning. CPE and HCG can continue to be used, but other hormones that have been used successfully to spawn other fish in captivity should be evaluated to determine if they are effective at inducing gonad maturation. Verification of ultrasound as a noninvasive technique for assessing gender is important and other noninvasive methods for assessing gender should be identified if ultrasound is not reliable. Finally, a comparative nutritional analysis of wild and captive Bonefish would identify any deficiencies that may be present in the diet of captive fish, and the diet could be adjusted to improve broodstock condition. Once identified, an optimal feed rate should be determined to avoid overfeeding and reduce costs for future projects.

CEI.

Changes in ambient photothermal conditions resulted in gonad development in the long-term group (23%), but not in the short-term group (0%). This indicates that Bonefish may need a long time to adapt to captivity before they respond to environmental cues. Reports from Atlantis Aquarium indicate that Bonefish were in the tank for one to two years before they unintentionally spawned volitionally under ambient conditions (D. Wert, personal communication). The stress of collecting the short-term group right before the

spawning season may have shut down gonad development for the entire season (Barton and Iwama 1991, Pankhurst and Van Der Kraak 1997, Zohar and Mylonas 2001), especially since they were collected with a seine net instead of hook-and-line.

Because the tanks were under ambient light and on flow-through from ambient water nearby, environmental cues were assumed to be identical to what the Bonefish would have experienced in the flats and creeks surrounding CEI. But it is possible that the water at the intake was not representative of the water conditions necessary to promote gonad development. For example, salinity measured during the course of these experiments declined from 35 ppt over the summer (May-September) to 28 ppt during the winter (October-January). Eleuthera does not have large surficial freshwater reserves that might account for this fluctuation (Army Corps of Engineers 2004). The source of the freshwater input is likely anthropogenic, from CEI itself and the marina located on the point of Cape Eleuthera, which represent the only development in the area. If this is a localized phenomenon and persistent annually, it does not disrupt the natural spawning of Bonefish as the PSA site (Danylchuk et al. 2011) – where pre-spawning activity still occurs (T. van Leeuwen, personal communication) and the successfully spawned fish was captured – which is located 1 km from the marina. If annual variation in salinity might be another environmental cue in tropical marine habitats (Boyer et al. 1999), along with photoperiod and temperature, it did not improve gonad maturation rates for the long-term group.

Bonefish were fed shrimp, squid, and queen conch offal. Squid and queen conch are not known prey items for Bonefish (Warmke and Erdman 1963, Bruger 1974, Crabtree et al. 1998), but squid is considered a valuable component for a broodstock diet (Izquierdo et al. 2001) and both have been used successfully in previous Bonefish studies at CEI (Murchie et al. 2009, Murchie et al. 2011a, Stein et al. 2012). Some of the Bonefish in those previous studies had gonad development (J. Shenker, personal communication), suggesting that the diet was adequate for this study.

No prior study has established a length-maturity relationship for Bonefish in The Bahamas, but in other locations in the Greater Caribbean maturation occurs at shorter lengths (Adams et al. 2007) than in the Florida Keys (Crabtree et al. 1997). The mean fork length for mature females in this study was 389 mm (n=9) with a minimum of 320 mm and maximum of 460 mm, and for mature males mean fork length was 371 mm (n=12) with a minimum of 340 mm and maximum of 413 mm. This is consistent with other Caribbean locations (Adams et al. 2007), but is obscured because the species of several mature fish was unknown and it is possible that some were *A. goreensis*, for which only basic information is available (Wallace and Tringali 2016, Rennert et al., 2018, Haak et al., 2018). Hybridization among Bonefish has been documented (Wallace and Tringali 2016) and is evidence that both species occasionally spawn together in the wild, suggesting that holding both species together was not an issue for spawning efforts in this study. Whether these hybrid offspring are viable has not been determined.

Most of the gonad development was observed in March towards the end of the study. Regional and species variation for peak spawning activity in Bonefish may exist (Crabtree et al. 1997, Donovan et al. 2015, A. Adams, personal communication) and previous observations from CEI staff indicate that the peak for this PSA is likely March (T. Van Leeuwen, personal communication). This could explain why the most gonadal development in the long-term group was seen in March and perhaps more gonad development would have been observed if the study had continued until the next full moon (March 31).

Three of the fish collected from the pre-spawn died, likely from the stress of repeated handling (Zohar and Mylonas 2001). This suggests that the other fish from this group were also stressed, which would have disrupted their reproductive hormones and the effect of hormone injections (Barton and Iwama 1991, Pankhurst and Van Der Kraak 1997). However, one fish from this group did spawn (Table 24), indicating that hormones might still be useful even with negative effects of handling stress.

DHP, which is used to induce ovulation in several fish species (Nagahama et al. 1983, Okamura et al. 2014), was also administered to female Bonefish in early December. DHP was not successful in inducing ovulation for the early December PSA group, but this was likely due to early application. DHP is a maturation-inducing hormone for several species of fish, but the cell membrane is most receptive to the hormone right before maturation (Nagahama 1997). DHP is only effective at inducing ovulation in eels when the oocyte is mature enough, typically when the diameter is greater than 700 µm (Kagawa et al. 1995). In the

late December PSA group, DHP likely induced ovulation for one fish (HOP6916), when the eggs were 971 µm. However, the spawn was not observed and no eggs were collected. DHP was going to be administered to the fish that spawned, which had eggs measuring 1,220 µm, but when the female was anaesthetized for the injection, it began releasing eggs and exogenous DHP was deemed unnecessary. This also suggests that exogenous DHP may not be necessary for inducing spawning in Bonefish. Because of the induced spawning with only CPE in late December, DHP was not administered to the female in late January, despite having eggs measuring 1,000 µm.

Future research should establish if seasonal variations in salinity exist on Eleuthera and other Bahamian islands where Bonefish spawning is observed and if so, does it serve as an environmental cue for gonad development and spawning in Bonefish. Determining a length-maturity relationship for *A. vulpes* in The Bahamas would provide a reference for future spawning work outside of the Florida Keys. Finally, once gonad development is achieved regularly, determining the range of diameters over which Bonefish oocytes are responsive to DHP will aid with spawning efforts.

Conclusion

In summary, photothermal manipulation and hormone injections, while successful in inducing gonad maturation and spawning in several species, were not as effective for Bonefish. Further work is needed to improve upon these initial efforts, but this work has shown that spawning techniques for temperate species may not be directly transferable to this species complex, expands upon the

research done with Bonefish in captivity, and provides new insight into Bonefish reproductive biology. Injections of CPE were used to induce spawning in Bonefish collected from a pre-spawn aggregation on Eleuthera, the first time that Bonefish have been spawned in captivity using hormones. Continuing this research into Bonefish spawning is important for a full understanding of their biology, to provide opportunities to work with unknown or poorly understood early life stages. In Chapter 2, development of eggs collected from the spawn and larvae that hatched from those eggs are imaged and described.

CHAPTER 2 – EMBRYOLOGY AND LARVAL TIME SERIES

Introduction

In general, completing a life history assessment for marine fish species is more difficult than freshwater species, especially if the marine species is a pelagic spawner. While each stage has its own research challenges, the egg and larval stages tend to be more difficult to study for several reasons. Collection and observation of these life stages in the field requires knowledge of spatiotemporal aspects of adult spawning as well as an understanding of the local oceanography. The egg stage of most warm-water, marine fish tends to be short in duration (Wellington and Victor 1989, Peters et al. 1998, Shanks 2009), so collection must occur quickly after spawning. Although the larval stage lasts longer, larvae are smaller and more delicate than the juvenile and adult stages, making them more difficult to collect alive and without damaging the fish (Post et al. 1997, Miller 2009).

Due to the difficulties in collecting and working with these life stages in the field, research into these life stages is lacking (Olafsen 2001, Miller 2009, Rønnestad et al. 2013). An alternative to field research is laboratory culture, which has been used successfully for describing eggs and early larval development in several eel species (Yamamoto 1981, Lokman & Young 2000, Oliveira & Hable 2010, Sørensen et al. 2016). This technique of using captive spawning to describe egg and larval development can be expanded to other

species, especially for species where opportunities for field research of early life stages are limited and that can be spawned in captivity.

Bonefish, like their relatives the eels (Anguilliformes), have early life stages that are difficult to study in the field. The general spatiotemporal characteristics of *Albula vulpes* spawning is thought to occur offshore at night, around full moons in October through March (Danylchuk et al. 2011). However, the precise spawning locations are unknown and spawning depth may be greater than 55 m (Aaron Adams, unpublished data). By the time the eggs have reached the surface, where they would be easier to collect, hatching may have already occurred or they might have been dispersed by the currents.

Bonefish share a unique larval form, known as a leptocephalus, with eels, tarpon, and ladyfish (Miller 2009). These larvae are difficult to collect alive because of their gelatinous bodies, which make them transparent and highly susceptible to damage during collection, especially using active collection methods (Miller 2009). Nearly all descriptions of Bonefish larvae come from late-stage or metamorphosing Bonefish leptocephali in the Gulf of California (Pfeiler 1981, Pfeiler 1984a, Pfeiler et al. 1988), but no description has been made of larvae during the first few weeks of development, including post-hatch or first-feeding.

Like eels, Bonefish early life stages would be easier to study and describe in a laboratory setting, using captive spawning to acquire eggs, compared to collecting these life stages in the wild. Fertilized eggs from unintentional spawns of Bonefish in the Ruins Lagoon Aquarium at the Atlantis Paradise Island

Bahamas (Nassau, New Providence) were collected and successfully hatched by staff, confirming that this approach is feasible. However, no images or formal descriptions were created (D. Wert, personal communication). As such, the early life stages of Bonefish remain undescribed.

In this study, adult Bonefish were collected from a pre-spawning aggregation (PSA) and induced to spawn in captivity with hormone injections at Cape Eleuthera Institute (CEI) in Eleuthera, The Bahamas. Eggs collected from the spawn were successfully fertilized, incubated until hatching, and reared for 56 hours. Egg and larval samples were collected to create a time series of early larval development. This is the first time that Bonefish ontogeny has been described from fertilization and these images and descriptions fill a crucial gap in the knowledge of Bonefish life history.

Methods

Spawning.

Fish were collected in Eleuthera from No Name Cut (Figure 5), a known Bonefish pre-spawning aggregation site, using hook-and-line on 31 December 2017 and transferred by boat to CEI in 100 L tubs filled with ambient water. Fish were held in a 13,000 L tank, under a pavilion with ambient light and flow-through water supply, with other fish also collected from No Name Cut. The female Bonefish was identified by eggs acquired from cannulation and received a 20 mg/mL injection of carp pituitary extract (CPE, Stoller Fisheries, Spirit Lake, IA) on 31 December 2017 and another on 1 January 2018. Eggs were 1,220 µm (n=7) after the second injection. The fish was anaesthetized with clove oil (0.8

mL/20 L) during cannulation and injections. On 2 January 2018, the female began releasing eggs while being anaesthetized before a planned third injection and she was rinsed to remove residual clove oil and then strip spawned into a clean metal bowl. The male Bonefish was identified by milt expressed when palpated abdominally and did not receive any hormone injections. The male was strip spawned onto the eggs in the bowl and eggs and milt were mixed before the addition of seawater to activate the sperm. Both fish were returned to the tank after being strip spawned.

Incubation, hatching, and rearing.

After fertilization, eggs were transferred from the bowl into a 6 L polycarbonate beaker (Figure 11), provided with aeration. Nine hours after fertilization, eggs were split evenly into another 6 L polycarbonate beaker, also with aeration, to reduce densities. The beakers were filled with ambient seawater collected from the flow-through system at CEI. For incubation, the water was pre-heated in 100 L tubs with 100 watt, submersible aquarium heaters (Aqueon, Franklin, WI) to between 23.9 and 25.6 °C for eggs. 25% water exchanges were conducted every hour to maintain water temperature. 50% water exchanges were conducted and eggs at the bottom of the beakers, which were assumed to be unfertilized or dead, were removed every six hours until hatching to maintain water quality. A subsample of the bottom eggs removed during the first exchange were put into another 6 L beaker for observation and to confirm they were not viable.

After hatching, larvae were reared in the same 6 L beakers. Rearing water was collected and heated in the same way, with water temperatures maintained between 23.9 and 25.6 °C. 25% water exchanges were conducted every hour to maintain water temperature. Mortalities were removed and 50% water exchanges were conducted every four hours to maintain water quality.

Microscopy.

Two subsamples of eggs were collected every hour, from each tank, from fertilization until hatching. Two subsamples of larvae were also collected at hatching (0 hours), 2 hours, 4 hours, and 6 hours post-hatch (hph) and then every four hours, until 56 hours for Tank 1 and 42 hours for Tank 2, when no larvae remained alive in the tanks.

For one of the subsamples, images were taken of eggs and larvae from each tank, at every hour, shortly after collection, with a 1.3MP Dino-Lite Edge microscope (Dino-Lite, Torrance, CA), using brightfield and darkfield illumination. After imaging, eggs and larvae were stored in cryovials and frozen at -20 °C for a separate project on lipid composition. Images of eggs were analyzed later using ImageJ (ver. 1.52a, Schneider at al. 2012) to measure oocyte diameter and chorion diameter, record developmental stage (see below for stage classification description), and count oil droplets. Images of larvae were also analyzed with ImageJ to measure notochord length (NL), total length (TL), area of the oil droplet, and area of the yolk.

The second subsample of eggs and larvae were fixed in 10% neutral buffered formalin. The fixed samples were later imaged, counted, and measured, using an

Olympus BX51 microscope and CellSens Standard (ver. 1.15, Olympus, Center Valley, PA). Measurements taken for eggs and larvae were the same as the measurements taken for the other subsample. Larval lengths, yolk areas, and oil areas were fitted with linear regressions. Regression analysis was done with Excel 2016 (Microsoft, Redmond, WA).

Classification.

Eggs were classified into the following developmental stages, used for the model organism Zebrafish (*Danio rerio*), modified from Kimmel et al. (1995): zygote, cleavage, blastula, gastrula, segmentation, and pharyngula. If a development stage could not be identified or if it was not possible to distinguish between two development stages, the egg was designated as Stage Undetermined (SU). The zygote stage was determined from the swelling of the chorion after fertilization until cleavage. The cleavage stage was determined from the observation of cell division in the zygote until the blastula. The blastula stage included the morula phase, where cells had grouped into a ball for blastulation, up to early epiboly. The gastrula stage was determined from observation of 50% epiboly until myomere segments began forming, which identified the segmentation stage. The pharyngula stage was determined by a post-anal tail and development of the eye.

Oil droplet coalescence was also recorded. The number of oil droplets present in the egg were divided into three classifications based on the number of oil droplets visible, similar to a classification used by Sørensen et al. (2016), which provided an indication of the level of oil droplet coalescence: multiple, few,

and single. “Multiple” consisted of seven or more oil droplets, typically small and loosely associated. “Few” was defined as two to six droplets, when they were larger in size and beginning to collect, similar to a berry cluster. Finally, “single” represented oil droplet coalescence. This method was used instead of taking the mean of oil droplets in an egg because it was not possible to determine if the number of oil droplets visible in pictures taken after collection represented the total number of oil droplets present and accurate counts of oil droplets in the fixed eggs was made difficult due to increase in opacity after fixation.

Results

Eggs.

Eggs were observed near the surface of the beakers throughout incubation, while some sank to the bottom and were removed. The remaining eggs successfully completed all six stages of development. Timing and duration of development stages was similar between the fresh and fixed eggs. Oil droplet coalescence did not occur until shortly before hatching for both groups. Eggs hatched simultaneously after 26 hours post-fertilization (hpf).

Eggs collected shortly after fertilization (0 hours) showed swelling chorions (1,417 ± 29 µm, n=5) relative to the oocyte (1,050 ± 14 µm, n=6), indicating successful activation (Figure 16, Table 26). The chorion continued to swell for three hours (Figure 17) until reaching a size between 1,709 to 2,080 µm (mean=1,951 ± 65 µm, n=88, Table 26), from 3 hours post-fertilization (hpf) to hatching (Figure 18, Figure 19, Figure 20, Figure 21). The diameter of the oocyte

ranged between 989 and 1,370 µm (mean=1,147 ± 83 µm, n=94) from 0 hpf to hatch (Figure 12, Figure 13, Table 26).

Of the eggs imaged at CEI (n=342), 261 (76%) were able to be staged (Figure 14) and 81 (24%) were SU. At 0 and 1 hpf, all eggs were identified in the zygote stage (mean=0.81 ± 0.75 hpf, n=31, Figure 16), indicating successful fertilization. The cleavage stage was observed at 2 hpf and last observed at 3 hpf (mean=2.3 ± 0.50 hpf, n=4, Figure 17). The first blastula was observed at 3 hpf and last observed at 9 hpf in Tank 1 (mean=5.6 ± 2.2 hpf, n=10, Figure 18) and 10 hpf in Tank 2 (mean=6.1 ± 2.6 hpf, n=11). The gastrula stage was first observed at 9 hpf for both tanks and last observed at 17 hpf in Tank 1 (mean=13 ± 2.2 hpf, n=39) and 18 hpf in Tank 2 (mean=13 ± 2.2 hpf, n=51, Figure 19). Segmentation was first observed at 15 hpf and last observed at 23 hpf in both tanks (Tank 1 mean=19 ± 2.4 hpf, n=40; Tank 2 mean=20 ± 2.2 hpf, n=52, Figure 20). The pharyngula stage was first observed at 24 hpf in Tank 1 (mean=24 ± 0.51 hpf, n=17, Figure 21) and 21 hpf in Tank 2 (mean=24 ± 1.1 hpf, n=15), before both tanks hatched at 26 hpf. Oil droplet coalescence did not begin until 21 hpf for both tanks (Figure 15, Figure 21). In Tank 1, 43% of eggs at 25 hpf had oil droplets that completely coalesced. In Tank 2, 20% of eggs at 24 hpf, and 44% of eggs at 25 hpf had oil droplets that completely coalesced.

Eggs that were fixed had chorions that measured between 1,057 to 2,100 µm (mean=1,914 ± 104.8 µm, n=382) and yolk diameters between 627 and 1,254 µm (mean=994 ± 124 µm, n=392) from post-fertilization to hatch (Figure 12, Figure 13, Table 27). Of the eggs that were fixed (n=393), 329 (84%) were able

to be staged (Figure 22) and 64 (16%) were SU. Zygotes were observed at 1 and 2 hpf (mean=1.6 ± 0.51 hpf, n=18, Figure 24). The cleavage stage was observed at 2 hpf and last observed at 5 hpf (mean=3.0 ± 1.2 hpf, n=7, Figure 25). The first blastula was observed at 2 hpf until 11 hpf in Tank 1 (mean=6.0 ± 2.1 hpf, n=35, Figure 26) and 10 hpf in Tank 2 (mean=5.8 ± 2.0 hpf, n=33). The gastrula stage was first observed at 6 hpf until 19 hpf in Tank 1 (mean=14 ± 2.6 hpf, n=91) and 18 hpf in Tank 2 (mean=13 ± 3.0 hpf, n=84, Figure 27). Segmentation was observed at 10 hpf until 23 hpf in Tank 1 (mean=20 ± 2.5 hpf, n=28, Figure 28) and 18 hpf to 23 hpf in Tank 2 (mean=21 ± 1.6 hpf, n=25). The pharyngula stage was first observed at 23 hpf in Tank 1 (mean=24 ± 0.75 hpf, n=24, Figure 29) and Tank 2 (mean=24 ± 0.78 hpf, n=21) until 26 hpf, when the eggs hatched. Oil droplet coalescence was seen in one egg at 8 hpf and again in two eggs in Tank 1 at 10 hpf, but did not occur again until 21 hpf in Tank 1 (Figure 23, Figure 29) and 20 hpf in Tank 2. In Tank 1, 14% of eggs at 23 hpf, 100% of eggs at 24 hpf, and 91% of eggs at 25 hpf had oil droplets that completely coalesced. In Tank 2, 40% of eggs at 23 hpf, 57% of eggs at 24 hpf, and 80% of eggs at 25 hpf had oil droplets that completely coalesced.

Egg mortality was substantial. While not directly quantified, it is estimated that >75% of the eggs died between fertilization and hatching and sank to the bottom of the beakers. All the sunken eggs were removed to maintain water quality. A subsample of bottom eggs was held and incubated in a separate beaker with aeration, but no development was seen after 22 hours, confirming that these eggs were dead or unfertilized.

Larvae.

Upon hatching at 26 hpf (0 hours post-hatch [hph], Figure 34), larvae were in a primitive form. No mouth was present and the eye was undeveloped. A large yolk was present in the abdomen along with an elongate oil droplet (Figure 35), which was completely coalesced in all larvae at 0 hph (Figure 34), at the anterior of the abdomen. Newly hatched larvae were oriented vertically in the beakers (Figure 11), with their heads oriented up presumably due to the large, anterior oil droplet. Larvae remained predominantly stationary, with sporadic darting movements being the main movement observed. Highest densities occurred at the surface (Figure 11), but larvae were observed throughout the water column. During larval development, yolk area decreased over time, while the oil droplet retained its size (Figure 36, Figure 37, Figure 38). Larvae from the final sampling periods (Figure 39) had grown significantly since hatching, developed unpigmented eyes, a visible gut but no mouth, visible otoliths, and were still transparent except for some posterior pigmentation at the end of the notochord.

Of the larvae imaged in the field, in Tank 1 larvae grew in length from 3,538 to 7,311 µm NL and 3,597 to 7,430 µm TL (n=11) over 56 hours (Figure 30). Oil droplet area decreased from 0.18 to 0.08 mm^2 (n=11) and yolk area decreased from 0.74 to 0.06 mm^2 (n=11) over the same time (Figure 31, Table 28). In Tank 2, larvae grew in length from 3,197 to 6,882 µm NL and 3,258 to 7,128 µm TL (n=8) over 40 hours (Figure 32). Oil droplet area decreased from 0.17 to 0.11 mm^2 (n=8) and yolk area decreased from 0.56 to 0.16 mm^2 (n=8) over the same time (Figure 33, Table 28).

Oil droplets were completely coalesced in all larvae at 0 hph (Figure 44). Of the fixed larvae, in Tank 1 larvae grew in length from 2,937 to 6,508 µm NL (n=75) and 3,133 to 6,554 µm TL (n=74) over 56 hours (Figure 40, Figure 46, Figure 48). Oil droplet area decreased from 0.22 to 0.068 mm^2 (n=76) and yolk area decreased from 0.82 to 0.24mm^2 (n=75) over the same time (Figure 41, Table 29). In Tank 2, larvae grew in length from 3,024 to 6,194 µm NL and 3,058 to 6,376 µm TL (n=149) over 42 hours (Figure 42, Figure 44, Figure 45, Figure 47). Oil droplet area decreased from 0.23 to 0.11 mm^2 and yolk area decreased from 0.81 to 0.25 mm^2 (n=148) over the same time (Figure 43, Table 29).

Discussion

Comparing the development of Bonefish eggs to other elopomorph species and non-elopomorph species, several differences are notable. Oil droplet coalescence in elopomorphs appears to occur shortly after fertilization (Lokman and Young 2000, Oliveira and Hable 2010, and Sørensen et al. 2016), as opposed to during oocyte maturation for many other species (Neidig et al. 2000, Patiño and Sullivan 2002, Arocha 2002), where it is often used as an indicator of egg maturation. Whether this delay in oil droplet coalescence is a true feature of elopomorph egg development or an artifact from hormone-induced spawning remains to be verified. Even restricting comparisons within hormone-induced spawning of other elopomorphs, the Bonefish eggs in this study did not have single oil droplets until immediately prior to hatching, much later than other elopomorphs. The delayed coalescence of the oil droplet needs to be confirmed through further study of fertilized eggs in captivity and the field. If oil droplet

coalescence takes longer in elopomorphs, developing new indicators of oocyte maturation would improve captive spawning prediction technique and success for elopomorphs.

Another variation between Bonefish development observed in this study and other elopomorphs is the shorter duration of Bonefish egg development. Several eel species take 36 to 48 hours before hatching (Oliveira and Hable 2010, Ahn et al. 2012, Sørensen et al. 2016), whereas the Bonefish eggs in this study hatched in only 26 hours. Some of the variation in hatching time may be attributable to the warmer rearing temperatures used in this study (23.9 °C to 25.5 °C) compared to the cooler temperatures used for most eels (18.2 °C to 23 °C, Sørensen et al. 2016), which reflects the cooler waters where eels spawn. Ahn et al. (2012) raised Japanese eels (*Anguilla japonica*) at temperatures ranging from 16 to 31 °C, and eggs kept at 25 °C, similar to the temperatures used in this study, hatched within 27-32 hours.

Since Bonefish have a tropical range, a higher temperature was used for incubating the eggs in this study, compared to temperate for eels. The precise location of Bonefish spawning is currently unknown, so no directly comparable water temperature data exist. However, pop-up archival tag data collected from Caribbean Reef Sharks (*Carcharhinus perezi*) in Exuma Sound, tagged along southern Eleuthera near the PSA site the broodstock were collected at, recorded water temperatures ranging between 22 to 26 °C in the upper 200 m during the spawning season (Shipley et al. 2017). Further research needs to be conducted to determine the range of temperatures Bonefish eggs experience in the wild, but

this study successfully incubated and hatched eggs in water between 23.9 and 25.5 °C and thus recommends this range for future work.

Two observations of eggs and larvae that were fixed compared to samples imaged at CEI was that opacity increased and sizes decreased for both eggs and larvae. Increased opacity after fixation has also been observed in hydrated oocytes from Weakfish (*Cynoscion regalis*) (Lowerre-Barbieri and Barbieri 1993). To improve images taken in the lab, unsuccessful attempts were made to clear the eggs using Serra's fluid, 100% glycerol, and a solution made of 30% saturated sodium borate, 70% DI water, and 0.1g trypsin powder. Glycerol worked in improving the visibility of one egg (Figure 49), but repeated attempts could not reproduce the effect for other eggs. The other two solutions did not improve the clarity of the eggs.

10% formalin was used to fix the eggs, and decreases in sizes were noted in both eggs and larvae. Oocytes of eggs that were fixed decreased in size by 10% on average. Similar shrinkage rates were also seen in hydrated oocytes of Weakfish (*Cynoscion regalis*) (Lowerre-Barbieri and Barbieri 1993). Chorions were 2% smaller on average after being fixed. Larvae that were fixed were 10% smaller in notochord length on average than larvae imaged at CEI, which is consistent with shrinkage rates observed for larvae of other species fixed in formalin (Hay 1982, Tucker and Chester 1984, Fey 1999).

The Bonefish larvae hatched at a larger size than Japanese Eel larvae (Yoshimatsu 2011), which may be a result of their respective larval durations. Bonefish have a larval duration of 41-71 days (Mojica et al. 1995), which is

shorter than the Japanese Eel (210 days, Zenimoto et al. 2011), and several tropical eel species (116-195 days, Marui et al. 2001). Hatching at a larger size may be necessary for Bonefish so that they can reach an appropriate and advantageous size before arriving at the settlement habitat in a shorter amount of time relative to eels.

Linear and logistic regressions were fitted to the notochord lengths for larvae, and linear models best described the variance in the data. Logistic growth was expected (Sørensen et al. 2016), with declining growth rates as endogenous energy was utilized, but the larvae likely did not survive long enough to approach the asymptote of their growth. Kurokawa et al. (1995) used two linear regressions to model the change in growth rate of post-hatch Japanese Eel larvae, finding that larvae grew faster for about the first week and before growth slowed down. Using the linear regressions to calculate daily growth rates, larvae imaged at CEI grew at 1.4 mm/day for Tank 1 (Figure 30) and 2.2 mm/day for Tank 2 (Figure 32) and for fixed larvae, growth rates were 0.9 mm/day for Tank 1 (Figure 40) and 1.5 mm/day for Tank 2 (Figure 42). The slower growth rate observed for the fixed larvae is likely due to shrinkage during fixation. The growth rates of the imaged larvae are consistent with leptocephali of other species, although variation exists between tropical and temperate species (Miller 2009) and the tropical distribution of Bonefish may explain the faster growth rates. An indication that larval growth rates must decline with age is demonstrated by calculating estimated sizes at the completion of the larval phase using the growth rates calculated. Using the minimum (41 days) and maximum (71 days) larval

durations a growth rate of 2.2 mm/day to calculate estimated size at recruitment, larvae should be between 94 and 160 mm, which is beyond the sizes of all but a few temperate eel species with much longer larval durations (Miller 2009). Using the conservative estimate of 1.4 mm/day would predict larvae between 61 and 103 mm when they move inshore to metamorphose and recruit as juveniles, which is still too large, but closer to Bonefish larval sizes reported by Pfeiler (1981, 1984b) in the Gulf of California and the size of pelagic larvae in the Bahamas (Mojica et al. 1995). Future larval rearing work should focus on measuring growth rates from hatching to exogenous feeding and exogenous feeding to metamorphosis to provide a clearer picture of larval Bonefish growth.

Comparison of the yolk and oil area indicates the yolk is preferentially used after hatching, which agrees with work on the Japanese Eel (Yoshimatsu 2011) and European Eel (Sørensen et al. 2016). For Bonefish, declines in yolk area were observed in both tanks for field and lab larvae while oil remained stable over time (Figure 31, Figure 33, Figure 41, Figure 43). Yolk consumption rates are similar to rates seen in other marine larvae (Johns et al. 1981, Fukuhara 1990, Williams et al. 2004). In the European Eel, oil droplet consumption coincided with mouth formation at 8 days post-hatch (dph) and was completely gone by 14 dph (Sørensen et al. 2016). Larvae in this study did not live long enough to compare oil utilization, but presumably the use of the oil droplet was being delayed, as seen in other fishes, as it may aid in buoyancy (Williams et al. 2004, Palińska-Żarska et al. 2014, Sørensen et al. 2016).

Possible causes for egg and larval mortality include water quality, stocking density, contamination, or insufficient energy reserves. Efforts were made to keep water quality in good condition, but dissolved oxygen (DO) may not have been high enough for eggs and larvae using just aeration. Oxygen demands for eggs and larvae of other sub-tropical species are higher than temperate species due largely to temperature (Houde and Schekter 1983, Houde 1989) and oxygen demand increases as the egg and larva develop (Houde and Schekter 1983). Preferably an oxygen line would also have been included with aeration, but was not available at CEI.

Frequent water changes were made to keep ammonia down, but no tests were run to determine ammonia concentrations and no sensitivity analysis of leptocephali to ammonia has been conducted to date. Larval sensitivity to ammonia has been shown in Red Drum (*Sciaenops ocellatus*), with early larval survival reduced significantly by concentrations of 0.3 mg/L, but tolerance improved with size (Holt and Arnold 1983). Due to the water source being coastal, background ammonia concentrations were also likely higher than what the larvae would normally experience in the oligotrophic waters offshore.

In conjunction with water quality, stocking density may have been artificially high. Captive culture of Japanese and European Eels stock larvae at densities between 40-100 larvae/L (Okamura et al. 2016, Sørensen et al. 2016) and estimates of larval density for this study fall within that range. However, larvae congregated at the surface of the water, possibly as a positive phototactic response (Figure 11). Positive phototaxis differs from the negative phototaxis

observed in Japanese Eels, although they also cluster at the surface when it is dark (Yamada et al. 2009). This clustering artificially inflated densities at the surface, increasing the chance of collision with other larvae, the tank, or the surface of the water, which can result in damage to the fragile larvae (A. Okamura, personal communication). Collisions between larvae were observed in the tank, but no damage was observed amongst the imaged or fixed larvae. However, dead larvae removed from the bottom of the tanks were not inspected for collision-related damage.

A leading contender for cause of mortality is biological contamination. The water used for rearing was not filtered or treated and as a result biological and bacterial contamination was likely high. One unidentified polychaete was found during larval rearing at 34 hph (Figure 50). Bacterial contamination of eggs (Figure 16) is also a common problem in aquaculture, requiring water treatment and disinfection to minimize bacterial contamination (Olafsen 2001). The use of nearshore water during this time period also exposed the larvae to higher densities of bacteria than they would normally encounter offshore (Azam et al. 1983) and possibly different bacterial assemblages as well.

Proper energy reserves are vital to ensure the larvae develops to the point that it can feed exogenously. It does not appear that the larvae in this study had insufficient reserves and died from starvation. The female used in this study produced the eggs in the wild on a diet that presumably includes everything the developing egg and larvae would need and the female likely would not have been at the PSA if the eggs had not received enough lipid material and matured.

Further the presence of yolk, although reduced, and oil, relatively unutilized, at 56 hph indicate that the larvae had not extinguished their reserves by this point, which is consistent with rates of yolk and oil utilization in eel species (Sørensen et al. 2016, Yoshimatsu 2011) and similar to utilization rates in other marine fishes (Johns et al. 1981, Fukuhara 1990, Williams et al. 2004). Whether or not the reserves were enough for the larvae to survive to exogenous feeding is undetermined.

Conclusion

This study represents the first time that Bonefish egg and larval development has been described. This information fills a crucial gap in the life history of Bonefish and further expands upon the developmental work done thus far on other elopomorph species. Bonefish eggs have delayed oil droplet coalescence and shorter incubation period compared to other elopomorphs. Larval development is similar, with yolk consumption and growth rates within the range of other elopomorphs. This work provides a baseline for our understanding of early Bonefish development and serves as a useful reference for future work in the field or in captivity. Future research efforts should include repeating this study at different water temperatures and assessing how this parameter affects timing of development of the egg and growth of the larvae. Also, repeating this study with filtered and treated water and recording water quality parameters like dissolved oxygen and ammonia to assess how these factors affect growth and mortality. Additionally, subsequent work should determine if delayed oil droplet coalescence is truly part of the development of elopomorph eggs. Further, larvae

must be reared to exogenous feeding to establish when this important transition occurs and what energy reserves are necessary to reach that point. Lastly feeding trials need to be conducted, as the diet of leptocephalus is still unknown. This work will be critical to understanding the early life stages of Bonefish and required for future work with Bonefish in captivity.

APPENDICES

Appendix A: Tables

Appendix B: Figures

Appendix A: Tables

Table 1. PIT tag numbers, fork lengths, weights, and species ID from Bonefish at FAU-HBOI collected from the Florida Keys. Fin clips from the two untagged fish, 538, 529, 543, and 504 did not have enough genetic material for identification. Fin clips were collected from the remaining three fish after the initial species ID and were not resubmitted for identification. N/A – no measurement taken.

PIT tag/ Fish ID	*FL (mm) Nov-16*	*FL (mm) Dec-17*	*Weight (g) Nov-16*	*Weight (g) Dec-17*	*Species ID*
-	N/A	N/A	677	N/A	-
-	N/A	N/A	1,173	N/A	*A. vulpes*
510	362	475	885	2,150	*A. vulpes*
514	330	449	645	1,310	*A. vulpes*
575	438	514	1,736	2,400	*A. vulpes*
572	451	N/A	1,636	N/A	*A. vulpes*
538	371	455	913	1,910	-
512	387	N/A	1,248	N/A	*A. vulpes*
561	394	N/A	1,103	N/A	*A. vulpes*
596	394	N/A	1,096	N/A	*A. vulpes*
529	419	486	1,148	2,360	-
543	438	N/A	1,467	N/A	-
523	324	527	586	2,215	*A. vulpes*
517	400	470	1,148	1,910	*A. vulpes*
577	337	N/A	649	N/A	*A. vulpes*
548	410	501	1,280	2,450	*A. vulpes*
593	451	523	1,810	2,970	*A. vulpes*
518	395	460	1,180	2,010	*A. vulpes*
504	378	465	997	1,830	-
532	416	505	1,321	2,530	*A. vulpes*
079	N/A	530	N/A	2,640	-
845	N/A	485	N/A	1,980	-
035	N/A	415	N/A	1,350	-

Table 2. PIT tag numbers, fork lengths, weights, and species ID from Bonefish at FAU-HBOI collected from Biscayne Bay. Fin clips were collected from the other 21 fish after the initial species ID and were not submitted for identification.

PIT tag/ Fish ID	*FL (mm) Feb-18*	*Weight (g) Feb-18*	*Species ID*
502	486	2,125	*A. vulpes*
567	495	2,095	*A. vulpes*
929	465	1,985	-
995	475	1,935	-
522	450	1,660	-
918	456	1,680	-
928	540	2,590	-
983	445	990	-
970	630	4,340	-
980	470	1,900	-
541	440	1,660	-
904	430	1,260	-
922	550	2,800	-
938	435	1,340	-
999	470	1,890	-
962	550	2,310	-
954	420	1,130	-
982	440	1,440	-
930	450	1,410	-
957	450	1,450	-
924	425	1,290	-
886	435	1,350	-
956	405	955	-

Table 3. Fish IDs, genders, gonad assessments, fork lengths, and hormone administrations on 2016 Nov-10 for the Florida Keys group at FAU-HBOI.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Hormones*	*Spawn*
502	Female	Eggs	486	No	No
567	Undetermined	-	495	No	No
510	Undetermined	-	362	No	No
514	Undetermined	-	330	No	No
575	Undetermined	-	438	No	No
572	Undetermined	-	451	No	No
538	Undetermined	-	371	No	No
512	Undetermined	-	387	No	No
561	Undetermined	-	394	No	No
596	Undetermined	-	394	No	No
529	Undetermined	-	419	No	No
543	Undetermined	-	438	No	No
523	Undetermined	-	234	No	No
517	Female	Eggs	400	No	No
577	Undetermined	-	337	No	No
548	Undetermined	-	410	No	No
593	Undetermined	-	451	No	No
518	Undetermined	-	395	No	No
504	Undetermined	-	378	No	No
532	Undetermined	-	416	No	No

Table 4. Fish IDs, genders, gonad assessments, ultrasound assessments, fork lengths, weights and hormone administrations on 2017 Jan-25 for the Florida Keys group at FAU-HBOI. Und. – undetermined, HCG – human chorionic gonadotropin.

Fish ID	*Gender*	*Gametes*	*Sonogram*	*FL (mm)*	*Weight (g)*	*Hormones*	*Spawn*
502	Female	-	Ovary	486	2,125	Ovaplant (75 µg)	No
567	Male	-	Testes	495	2,095	1,000 IU HCG	No
510	Female	-	Ovary	362	885	No	No
514	Male	-	Testes	330	645	No	No
575	Male	-	Testes	438	1,736	No	No
538	Male	-	Testes	371	913	1,000 IU HCG	No
512	Female	-	Ovary	387	1,248	No	No
561	Male	-	Testes	394	1,103	1,000 IU HCG	No
596	Male	Sperm	Testes	394	1,096	1,000 IU HCG	No
529	Female	-	Ovary	419	1,148	Control	No
543	Und.	-	-	438	1,467	No	No
523	Und.	-	-	324	586	No	No
517	Female	-	Ovary	400	1,148	Ovaplant (75 µg)	No
577	Und.	-	-	337	649	No	No
548	Female	-	Ovary	410	1,280	No	No
593	Female	-	Ovary	451	1,810	Control	No
518	Female	-	Ovary	395	1,180	No	No
504	Male	-	Testes	378	997	1,000 IU HCG	No
532	Male	-	Testes	416	1,321	1,000 IU HCG	No

Table 5. Fish IDs, genders, gonad assessments, fork lengths, and hormone administrations on 2017 Nov-04 for the Florida Keys group at FAU-HBOI. N/A – not cannulated or measured.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Hormones*	*Spawn?*
510	Female	-	349	No	No
514	Male	-	318	No	No
575	Male	-	514	No	No
538	Male	-	455	No	No
529	Female	-	486	No	No
523	Undetermined	-	527	No	No
517	Female	-	470	No	No
548	Female	-	501	No	No
593	Female	-	523	No	No
518	Female	-	395	No	No
504	Male	-	465	No	No
532	Male	-	505	No	No
079	Undetermined	-	530	No	No
845	Undetermined	N/A	N/A	No	No
035	Undetermined	N/A	N/A	No	No

Table 6. Fish IDs, genders, gonad assessments, fork lengths, weights, and hormone administrations on 2017 Dec-04 for the Florida Keys group at FAU-HBOI. Lengths and weights were taken again at this time. Und. – undetermined, HCG – human chorionic gonadotropin, CPE – carp pituitary extract.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Weight (g)*	*Hormones*	*Spawn*
510	Female	-	475	2,150	No	No
514	Male	-	449	1,310	No	No
575	Male	-	514	2,400	1,000 IU HCG	No
538	Male	-	455	1,910	1,000 IU HCG	No
529	Female	-	486	2,360	20 mg CPE	No
523	Und.	-	527	2,215	No	No
517	Female	-	470	1,910	1,000 IU HCG	No
548	Female	-	501	2,450	20 mg CPE	No
593	Female	-	523	2,970	30 mg CPE	No
518	Female	-	460	2,010	No	No
504	Male	-	465	1,830	1,000 IU HCG	No
532	Male	-	505	2,530	1,000 IU HCG	No
079	Und.	-	530	2,640	1,000 IU HCG	No
845	Und.	-	485	1,980	No	No
035	Und.	-	415	1,350	No	No

Table 7. Fish IDs, gender, gonad assessment, fork lengths, weights, and hormone administration on 2017 Dec-31 for the Florida Keys group at FAU-HBOI. Und. – undetermined, CPE – carp pituitary extract.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Weight (g)*	*Hormones*	*Spawn*
510	Female	-	475	2,150	No	No
514	Male	-	449	1,310	No	No
575	Male	-	514	2,400	No	No
538	Male	-	455	1,910	No	No
529	Female	-	486	2,360	No	No
523	Und.	-	527	2,215	No	No
517	Female	-	470	1,910	No	No
548	Female	-	501	2,450	No	No
593	Female	-	523	2,970	No	No
518	Female	-	460	2,010	No	No
504	Male	-	465	1,830	No	No
532	Male	-	505	2,530	No	No
079	Female	Eggs	530	2,640	20 mg CPE	No
845	Und.	-	485	1,980	No	No
035	Und.	-	415	1,350	No	No

Table 8. Fish IDs, genders, gonad assessments, fork lengths, weights, and hormone administrations on 2018 Jan-31 for the Florida Keys group at FAU-HBOI. Fish 079 received a second injection of CPE on 2018 Feb-04. Und. – undetermined, CPE – carp pituitary extract.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Weight (g)*	*Hormones*	*Spawn*
510	Female	-	475	2,150	10 mg CPE	No
514	Male	-	449	1,310	10 mg CPE	No
575	Male	-	514	2,400	10 mg CPE	No
538	Male	-	455	1,910	10 mg CPE	No
529	Female	-	486	2,360	10 mg CPE	No
523	Und.	-	527	2,215	10 mg CPE	No
517	Female	-	470	1,910	10 mg CPE	No
548	Female	-	501	2,450	10 mg CPE	No
593	Female	-	523	2,970	10 mg CPE	No
518	Female	-	460	2,010	10 mg CPE	No
504	Male	-	465	1,830	10 mg CPE	No
532	Male	-	505	2,530	10 mg CPE	No
079	Female	Eggs 200-300 µm	530	2,640	20 mg CPE 20 mg CPE	No
845	Und.	-	485	1,980	10 mg CPE	No
035	Und.	-	415	1,350	10 mg CPE	No

Table 9. Fish IDs, genders, gonad assessments, fork lengths, weights, and hormone administrations on 2018 Feb-28 for the Florida Keys group at FAU-HBOI. Und. – undetermined, HCG – human chorionic gonadotropin, CPE – carp pituitary extract.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Weight (g)*	*Hormones*	*Spawn*
510	Female	-	475	2,150	20 mg CPE	No
514	Male	-	449	1,310	20 mg CPE	No
575	Male	-	514	2,400	1,500 IU HCG	No
538	Male	Sperm	455	1,910	1,500 IU HCG	No
529	Female	-	486	2,360	20 mg CPE	No
523	Und.	-	527	2,215	20 mg CPE	No
517	Female	-	470	1,910	20 mg CPE	No
548	Female	-	501	2,450	20 mg CPE	No
593	Female	-	523	2,970	20 mg CPE	No
518	Female	-	460	2,010	1,500 IU HCG	No
504	Male	-	465	1,830	1,500 IU HCG	No
532	Male	-	505	2,530	1,500 IU HCG	No
079	Female	Eggs 200 µm	530	2,640	20 mg CPE	No
845	Und.	-	485	1,980	20 mg CPE	No
035	Und.	-	415	1,350	20 mg CPE	No

Table 10. Fish IDs, genders, gonad assessments, fork lengths, weights, and hormone administrations on 2018 Jun-07 for the Florida Keys group at FAU-HBOI. Fish 956 was added to the tank and injected on 2018 Jun-08. Und. – undetermined, HCG – human chorionic gonadotropin, CPE – carp pituitary extract.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Weight (g)*	*Hormones*	*Spawn*
510	Female	-	475	2,150	40 mg CPE	No
514	Male	-	449	1,310	60 mg CPE	No
575	Male	-	514	2,400	50 mg CPE	No
538	Male	-	455	1,910	1,500 IU HCG	No
529	Female	-	486	2,360	60 mg CPE	No
517	Female	-	470	1,910	20 mg CPE	No
548	Female	-	501	2,450	25 mg CPE	No
593	Female	-	523	2,970	Control	No
518	Female	Eggs 100 µm	460	2,010	1,500 IU HCG	No
532	Male	-	505	2,530	50 mg CPE	No
079	Female	Eggs 300-375 µm	530	2,640	50 mg CPE	No
845	Und.	-	485	1,980	60 mg CPE	No
035	Und.	-	415	1,350	1,500 IU HCG	No
942	Und.	-	556	2,275	25 mg CPE	No
*956**	Und.	-	405	955	25 mg CPE	No

Table 11. Fish IDs, genders, gonad assessments, fork lengths, weights, and hormone administrations on 2018 Jun-28 for the Florida Keys group at FAU-HBOI. Und. – undetermined, HCG – human chorionic gonadotropin, CPE – carp pituitary extract.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Weight (g)*	*Hormones*	*Spawn*
510	Female	-	475	2,150	40 mg CPE	No
514	Male	-	449	1,310	60 mg CPE	No
575	Male	-	514	2,400	50 mg CPE	No
538	Male	-	455	1,910	1,500 IU HCG	No
529	Female	-	486	2,360	60 mg CPE	No
548	Female	-	501	2,450	25 mg CPE	No
593	Female	-	523	2,970	Control	No
518	Female	Eggs 100 µm	460	2,010	1,500 IU HCG	No
532	Male	-	505	2,530	50 mg CPE	No
079	Female	Eggs 300-375 µm	530	2,640	50 mg CPE	No
845	Und.	-	485	1,980	60 mg CPE	No
035	Und.	-	415	1,350	1,500 IU HCG	No
942	Und.	-	556	2,275	25 mg CPE	No
956	Und.	-	405	955	25 mg CPE	No

Table 12. Fish IDs, genders, gonad assessments, fork lengths, weights, and hormone administrations on 2018 Jul-27 for the Florida Keys group at FAU-HBOI. Und. – undetermined, HCG – human chorionic gonadotropin, CPE – carp pituitary extract.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Weight (g)*	*Hormones*	*Spawn*
510	Female	-	475	2,150	40 mg CPE	No
514	Male	-	449	1,310	60 mg CPE	No
538	Male	-	455	1,910	1,500 IU HCG	No
529	Female	-	486	2,360	60 mg CPE	No
548	Female	-	501	2,450	25 mg CPE	No
593	Female	-	523	2,970	Control	No
532	Male	-	505	2,530	50 mg CPE	No
079	Female	-	530	2,640	50 mg CPE	No
845	Und.	-	485	1,980	60 mg CPE	No
035	Und.	-	415	1,350	1,500 IU HCG	No
942	Und.	-	556	2,275	25 mg CPE	No
956	Und.	-	405	955	25 mg CPE	No

Table 13. Fish IDs, genders, gonad assessments, fork lengths, weights, and hormone administrations on 2018 Feb-01 for the Biscayne Bay group at FAU-HBOI. Fish 928 and 970 received second injections on 2018 Feb-02. Und. – undetermined, HCG – human chorionic gonadotropin, CPE – carp pituitary extract.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Weight (g)*	*Hormone*	*Spawn*
929	Und.	-	465	1,985	10 mg CPE	No
995	Male	Sperm	475	1,935	10 mg CPE	No
522	Male	Sperm	450	1,660	1,000 IU HCG	No
918	Male	Sperm	456	1,680	1,000 IU HCG	No
*928**	Female	Eggs 300 µm	540	2,590	60 mg CPE 60 mg CPE	No
983	Und.	-	445	990	1,000 IU HCG	No
*970**	Female	Eggs 500-600 µm	630	4,340	110 mg CPE 120 mg CPE	No
980	Female	Eggs 250-300 µm	470	1,900	10 mg CPE	No
541	Und.	-	440	1,660	1,000 IU HCG	No
904	Male	Sperm	430	1,260	1,000 IU HCG	No
922	Male	Sperm	550	2,800	1,000 IU HCG	No
938	Und.	-	435	1,340	1,000 IU HCG	No
999	Female	Eggs 100 µm	470	1,890	10 mg CPE	No
962	Und.	-	550	2,310	1,000 IU HCG	No
954	Und.	-	420	1,130	10 mg CPE	No
982	Male	Sperm	440	1,440	10 mg CPE	No
930	Und.	-	450	1,410	10 mg CPE	No
957	Und.	-	450	1,450	10 mg CPE	No
924	Und.	-	425	1,290	10 mg CPE	No
886	Und.	-	435	1,350	10 mg CPE	No

Table 14. Fish IDs, genders, gonad assessments, fork lengths, weights, and hormone administrations on 2018 Mar-01 for the Biscayne Bay group at FAU-HBOI. Und. – undetermined, HCG – human chorionic gonadotropin, CPE – carp pituitary extract.

Fish ID	*Gender*	*Gametes*	*FL (mm)*	*Weight (g)*	*Hormone*	*Spawn*
929	Und.	-	465	1,985	15 mg CPE	No
995	Male	-	475	1,935	1,500 IU HCG	No
522	Male	-	450	1,660	1,500 IU HCG	No
918	Male	-	456	1,680	1,500 IU HCG	No
928	Female	-	540	2,590	20 mg CPE	No
983	Male	Sperm	445	990	1,500 IU HCG	No
970	Female	-	630	4,340	60 mg CPE	No
980	Female	-	470	1,900	20 mg CPE	No
541	Und.	-	440	1,660	1,500 IU HCG	No
904	Male	-	430	1,260	1,500 IU HCG	No
922	Male	-	550	2,800	1,500 IU HCG	No
938	Male	Sperm	435	1,340	1,500 IU HCG	No
999	Female	-	470	1,890	15 mg CPE	No
962	Und.	-	550	2,310	1,500 IU HCG	No
954	Und.	-	420	1,130	No	No
982	Male	-	440	1,440	1,500 IU HCG	No
930	Und.	-	450	1,410	1,500 IU HCG	No
957	Und.	-	450	1,450	No	No
924	Und.	-	425	1,290	No	No
886	Und.	-	435	1,350	No	No

Table 15. Initial gonad status (left column) and gonad status after receiving an injection (top row) of carp pituitary extract (CPE). Measurements are off egg diameters. Dosages are given in mg/kg and the number in parentheses is the number of occurrences. Gray boxes indicate no change in status.

CPE	No gametes	Sperm	0-100 µm	101-200 µm	201-300 µm	301-400 µm	401-500 µm	501-600 µm	601-700 µm	701-800 µm	801-900 µm	901-1,000 µm	1,001-1,100 µm	1,101-1,200 µm	1,200-1,300 µm	Ovulation
	3 mg/kg (1)															
	5 mg/kg (12)															
	6 mg/kg (1)															
	10 mg/kg (16)															
	20 mg/kg (11)															
	25 mg/kg (4)															
	30 mg/kg (2)															
No gametes	40 mg/kg (2)	5 mg/kg (1)														
	5 mg/kg (1)															
Sperm	10 mg/kg (1)															
001-100 µm																
101-200 µm	5 mg/kg (1)				10 mg/kg (1)	10 mg/kg (1)										
					10 mg/kg (2)											
201-300 µm	5 mg/kg (1)				25 mg/kg (1)											
301-400 µm	20 mg/kg (1)					25 mg/kg (2)										
401-500 µm																
501-600 µm								25 mg/kg (1)	30 mg/kg (1)							
601-700 µm												10 mg/kg (1)				
701-800 µm													10 mg/kg (1)			
801-900 µm																
901-1,000 µm																
1,001-1,100 µm													30 mg/kg (2)			
1,101-1,200 µm																
1,200-1,300 µm																20 mg/kg (1)

Table 16. Initial gonad status (left column) and gonad status after receiving an injection (top row) of human chorionic gonadotropin (HCG). Measurements are of egg diameters. Dosages are given in IU and the number in parentheses is the number of occurrences. Gray boxes indicate no change in status. Diameters greater than 200 µm were excluded as no fish with eggs that size received injections or produced eggs that big after an injection.

HCG	No gametes	Sperm	0-100 µm	101-200 µm
	1,000 IU (15)			
No gametes	1,500 IU (6)	1,000 IU (2)	1,500 IU (1)	1,000 IU (1)
	1,000 IU (4)			
Sperm	1,500 IU (1)			
001-100 µm			1,500 IU (1)	
101-200 µm	1,500 IU (1)			

Table 17. Dart tags, tank assignments, fork lengths, weights, and species IDs for the Bonefish in the long-term group at CEI. Fish with two dart tags listed were re-tagged after the first one (listed first) fell out. N/A – measurement not taken.

Dart tag	*Tank*	*Fork Length (mm)*	*Weight (g)*	*Species ID*
HOP6334	1	350	539	*A. goreensis*
HOP6336		325	410	*A. goreensis*
HOP6337		330	447	*A. vulpes*
HOP6355		320	382	*A. goreensis*
HOP6373		309	314	*A. vulpes*
HOP6374		363	505	*A. vulpes*
HOP6383		374	576	*A. vulpes*
HOP6384/4404		294	282	*A. vulpes*
HOP6385		308	302	*A. vulpes*
HOP6846		N/A	N/A	*A. vulpes*
HOP6897		N/A	N/A	*A. vulpes*
HOP6328	2	367	597	*A. goreensis*
HOP6330		342	430	*A. goreensis*
HOP6335		355	510	*A. goreensis*
HOP6352		364	527	*A. vulpes*
HOP6354/4948		375	675	*A. vulpes*
HOP6372		325	N/A	*A. vulpes*
HOP6380		350	N/A	*A. vulpes*
HOP6381		363	533	*A. vulpes*
HOP6386/4941		350	509	*A. vulpes*
HOP6894		N/A	N/A	*A. vulpes*
HOP6849		N/A	N/A	*A. vulpes*

Table 18. Dart tags, tank assignments, fork lengths, weights, and species IDs for the Bonefish in the long-term group at CEI. Fish with two dart tags listed were re-tagged after the first one (listed first) fell out. HOP6375/5534 was a hybrid. N/A – measurement not taken.

Dart tag	*Tank*	*Fork Length (mm)*	*Weight (g)*	*Species ID*
HOP6329	3	330	435	*A. vulpes*
HOP6333		375	556	*A. goreensis*
HOP6370		385	N/A	*A. vulpes*
		310	350	*A. vulpes x*
HOP6375/5534				*A. goreensis*
HOP6376		369	583	*A. vulpes*
HOP6377/3419		349	508	*A. vulpes*
HOP6378		378	679	*A. vulpes*
HOP6379		355	560	*A. vulpes*
HOP6387/4322		295	363	*A. vulpes*
HOP6842/4349		N/A	N/A	*A. vulpes*
HOP6898		N/A	N/A	*A. vulpes*
HOP6331/3420	4	337	471	*A. goreensis*
HOP6351/5533		386	648	*A. vulpes*
HOP6353/3403		365	564	*A. vulpes*
HOP6358		385	666	*A. vulpes*
HOP6367		330	N/A	*A. vulpes*
HOP6368		348	N/A	*A. vulpes*
HOP6369		376	N/A	*A. vulpes*
HOP6371		460	1,041	*A. vulpes*
HOP6382/5517		367	585	*A. vulpes*
HOP6891		N/A	N/A	*A. vulpes*
HOP6896/4350		N/A	N/A	*A. vulpes*

Table 19. Dart tags, gametes collected, fork lengths, and weights for the early December PSA group. N/A – measurement not taken.

Dart tag	*Gametes*	*Fork length (mm)*	*Weight (g)*
HOP6824	Sperm	345	413
HOP6825	Eggs	425	823
HOP6826	Eggs	452	1,012
HOP6827	-	370	N/A
HOP6828	Sperm	364	449
HOP6829	Sperm	413	806
HOP6830	Eggs	355	595
HOP6850	-	320	N/A
HOP6882	Sperm	N/A	N/A
HOP6887	-	360	N/A
HOP6888	Eggs	360	547
HOP6889	-	N/A	N/A
HOP6890	-	390	N/A
HOP6893	Sperm	354	N/A
HOP6895	Sperm	N/A	N/A

Table 20. Dart tags, gametes collected, and fork lengths for the late December PSA group. No weights were taken. N/A – measurement not taken.

Dart tag	*Gametes*	*Fork length (mm)*
HOP6831	-	344
HOP6832	-	N/A
HOP6885	-	N/A
HOP6913	Sperm	370
HOP6914	Eggs	320
HOP6915	Eggs	390
HOP6916	Eggs	320
HOP6918	-	N/A
HOP6919	-	390
HOP6921	-	380

Table 21. Dart tags, gametes collected, and fork lengths for the January PSA group. No weights were taken. N/A – measurement not taken.

Dart tag	*Gametes*	*Fork length (mm)*
HOP6901	Sperm	N/A
HOP6902	Sperm	400
HOP6903	Sperm	340
HOP6904	Sperm	360
HOP6905	Sperm	388
-	Sperm	350
-	Sperm	310
-	-	390
-	-	326
-	-	308

Table 22. Dart tags and gametes collected from palpating or cannulation Bonefish in the short-term group at CEI. N/A – gonad development was not checked on that date.

Dart tag	*12/29/2017*	*12/30/2017*	*1/30/2018*
HOP6844	-	N/A	-
HOP6841	-	N/A	-
HOP6833	-	N/A	-
HOP6917	-	N/A	-
HOP6801	-	N/A	-
HOP6867	N/A	-	-
HOP6819	N/A	-	-
HOP6802	N/A	-	-
HOP6836	N/A	-	-
HOP6822	N/A	-	-
HOP6804	N/A	-	-
HOP6813	N/A	N/A	-
HOP6817	N/A	N/A	-
HOP6810	N/A	N/A	-
HOP6805	N/A	N/A	-
HOP6809	N/A	N/A	-
HOP6835	N/A	N/A	-
HOP6818	N/A	N/A	-
HOP6840	N/A	N/A	-
HOP6808	N/A	N/A	-
HOP6803	N/A	N/A	-
HOP6807	N/A	N/A	-
HOP6838	N/A	N/A	-
HOP6816	N/A	N/A	-
HOP6814	N/A	N/A	-
HOP6815	N/A	N/A	-
HOP6821	N/A	N/A	-
HOP6806	N/A	N/A	-
HOP6811	N/A	N/A	-
HOP6812	N/A	N/A	-
HOP6857	N/A	N/A	-

Table 23. Hormone injections administered to the early December PSA group. Females received CPE and DHP while males received HCG. Three males did not receive hormone injections because they were already producing sperm. HCG – human chorionic gonadotropin, CPE – carp pituitary extract, DHP – 17α,20β-dihydroxy-4-pregnen-3-one.

Dart tag	*Gender*	*Dec 4th*	*Dec 5th*	*Dec 6th*	*Dec 7th*	*Spawn*
HOP6824	Male	1,000 IU HCG				No
HOP6825	Female	10 mg CPE	2 mg DHP	2 mg DHP		No
HOP6826	Female	10 mg CPE	2 mg DHP	2 mg DHP		No
HOP6828	Male	1,000 IU HCG				No
HOP6829	Male	1,000 IU HCG				No
HOP6830	Female	5 mg CPE	2 mg DHP	5 mg DHP		No
HOP6882	Male					No
HOP6888	Female		5 mg CPE		1 mg DHP	No
HOP6893	Male					No
HOP6895	Male					No

Table 24. Hormone injections (top line) administered to the late December PSA group and average egg diameters (bottom line). Females received CPE and DHP and the male did not receive any hormone injections. CPE – carp pituitary extract, DHP – 17α,20β-dihydroxy-4-pregnen-3-one.

Dart tag	*Gender*	*Dec 30th*	*Dec 31st*	*Jan 1st*	*Spawn*
HOP6913	Male				Yes
HOP6915	Female		20 mg CPE	20 mg CPE	Yes
				1,220 µm	
HOP6916	Female	10 mg CPE	10 mg CPE	2 mg DHP	No
		675 µm	971 µm		

Table 25. Hormone injections (top line) administered to female fish from the early and late December PSA groups and average egg diameters (bottom line). The male was collected in January and did not receive an injection because it was already producing sperm. Females received CPE injections and the male received HCG injections. HCG – human chorionic gonadotropin, CPE – carp pituitary extract.

Dart tag	*Gender*	*Jan 29th*	*Jan 30th*	*Jan 31st*	*Feb 1st*	*Feb 2nd*	*Spawn*
HOP6830	Female	10 mg CPE		10 mg CPE			No
HOP6913	Male	1,000 IU HCG					No
HOP6914	Female	10 mg CPE		30 mg CPE	30 mg CPE	30 mg CPE	No
		700 µm		1,000 µm	1,000 µm	1,000 µm	
HOP6915	Female	10 mg CPE		10 mg CPE			No

Table 26. Hours post-fertilization (hpf), tank, n, oocyte and chorion diameters of eggs imaged at CEI, and the water temperature when they were collected. Measurements could not be taken at 11 hpf (Tank 2) and 12 hpf (Tank 1 and 2).

Post-fertilization	*Tank*	*n*	*Oocyte dia. ± SD (µm)*	*n*	*Chorion dia. ± SD (µm)*	*Temp. (°C)*
0 hour	1	6	1050 ± 14	5	1417 ± 29	25.0
1 hour	1	3	1166 ± 12	3	1763 ± 46	25.0
2 hours	1	5	1081 ± 145	5	1871 ± 63	23.9
3 hours	1	4	1198 ± 39	4	1963 ± 29	23.9
4 hours	1	4	1146 ± 45	4	1955 ± 42	25.0
5 hours	1	2	1183 ± 52	2	1953 ± 11	25.5
6 hours	1	4	1087 ± 40	4	1933 ± 18	25.5
7 hours	1	2	1108 ± 8	2	1969 ± 21	24.4
8 hours	1	5	1229 ± 34	5	1953 ± 106	25.5
9 hours	1	5	1223 ± 56	5	2038 ± 27	25.0
	2	4	1074 ± 42	4	1881 ± 52	25.0
10 hours	1	2	1136 ± 98	2	1960 ± 55	23.9
	2	1	1152	1	1916	23.9
11 hours	1	2	1114 ± 54	2	1980 ± 35	24.4
	2	0	N/A	0	N/A	24.4
12 hours	1	0	N/A	0	N/A	23.9
	2	0	N/A	0	N/A	23.9
13 hours	1	1	1250	1	2002	25.5
	2	1	1205	1	1947	25.5
14 hours	1	2	1217 ± 3	2	1993 ± 23	25.5
	2	2	1208 ± 38	2	1953 ± 54	25.5
15 hours	1	2	1170 ± 8	2	1945 ± 49	24.4
	2	6	1219 ± 85	6	1970 ± 35	24.4
16 hours	1	2	1177 ± 45	2	1967 ± 21	24.4
	2	1	1226	1	1947	24.4
17 hours	1	1	1211	1	2052	24.4
	2	1	1215	1	1943	24.4
18 hours	1	2	1139 ± 30	2	1952 ± 12	25.0
	2	2	1205 ± 21	2	2007 ± 26	25.0
19 hours	1	2	1172 ± 39	2	1989 ± 17	24.4
	2	1	1047	1	1935	24.4
20 hours	1	2	1117 ± 59	2	1946 ± 8	24.4
	2	2	1155 ± 58	2	1962 ± 27	24.4
21 hours	1	2	1065 ± 11	2	1968 ± 4	24.4
	2	1	1181	1	1973	24.4
22 hours	1	2	1076 ± 75	2	1985 ± 3	25.0
	2	2	1122 ± 30	2	1980 ± 15	25.0
23 hours	1	3	1137 ± 41	3	1959 ± 30	25.0
	2	1	989	1	1933	25.0
24 hours	1	1	1316	1	1921	25.5
	2	1	1292	1	2001	25.5
25 hours	1	1	826	1	1912	24.4
	2	1	978	1	1967	24.4

Table 27. Hours post-fertilization (hpf), tank, n, oocyte and chorion diameters of fixed eggs, and the water temperature when the eggs were collected.

Post-fertilization	*Tank*	*n*	*Oocyte dia. ± SD (µm)*	*n*	*Chorion dia. ± SD (µm)*	*Temp. (°C)*
1 hour	1	10	1111 ± 46.53	5	1626 ± 58.08	25.0
2 hours	1	16	1028 ± 39.69	16	1929 ± 70.55	23.9
3 hours	1	5	1081 ± 35.41	5	1768 ± 380.5	23.9
4 hours	1	9	1057 ± 31.67	9	1918 ± 19.69	25.0
5 hours	1	18	1070 ± 21.47	17	1923 ± 26.53	25.5
6 hours	1	18	1042 ± 17.95	18	1910 ± 67.28	25.5
7 hours	1	6	1048 ± 13.57	6	1973 ± 35.51	24.4
8 hours	1	10	1042 ± 153.1	9	1798 ± 239.0	25.5
9 hours	1	7	1075 ± 40.76	7	1972 ± 17.89	25.0
	2	8	1020 ± 61.69	8	1900 ± 21.58	25.0
10 hours	1	12	923 ± 112	12	1921 ± 14.34	23.9
	2	13	981 ± 73.1	13	1917 ± 13.24	23.9
11 hours	1	10	892 ± 121	10	1951 ± 18.42	24.4
	2	8	895 ± 142	8	1701 ± 390.5	24.4
12 hours	1	8	1079 ± 13.77	8	1911 ± 76.06	23.9
	2	4	1024 ± 18.30	4	1954 ± 44.49	23.9
13 hours	1	13	1055 ± 54.22	13	1973 ± 67.15	25.5
	2	20	931 ± 37.5	20	1907 ± 18.10	25.5
14 hours	1	20	1048 ± 31.44	20	1926 ± 17.58	25.5
	2	9	1141 ± 63.81	9	1947 ± 37.03	25.5
15 hours	1	18	1078 ± 35.55	18	1931 ± 20.87	24.4
	2	13	1084 ± 38.59	13	1886 ± 94.61	24.4
16 hours	1	6	1061 ± 14.23	6	1914 ± 16.20	24.4
	2	8	1070 ± 17.43	7	1932 ± 16.74	24.4
17 hours	1	9	1043 ± 100.5	9	1947 ± 25.52	24.4
	2	5	1069 ± 18.07	5	1944 ± 14.87	24.4
18 hours	1	8	1033 ± 22.92	7	1912 ± 19.08	25.0
	2	9	1046 ± 38.10	9	1949 ± 13.22	25.0
19 hours	1	5	1039 ± 20.11	5	1948 ± 27.31	24.4
	2	3	1042 ± 17.66	3	1925 ± 19.96	24.4
20 hours	1	3	957 ± 13.7	3	1969 ± 23.45	24.4
	2	6	1034 ± 17.88	6	1934 ± 11.42	24.4
21 hours	1	10	1008 ± 31.79	10	1926 ± 24.96	24.4
	2	3	1035 ± 14.23	3	1927 ± 14.09	24.4
22 hours	1	3	1001 ± 34.92	3	1969 ± 86.92	25.0
	2	5	979 ± 22.5	5	1921 ± 10.16	25.0
23 hours	1	7	899 ± 51.8	7	1946 ± 15.79	25.0
	2	10	880 ± 66.2	9	1920 ± 11.50	25.0
24 hours	1	9	720 ± 21.8	9	1958 ± 41.77	25.5
	2	7	795 ± 11.7	7	1855 ± 232.5	25.5
25 hours	1	11	691 ± 27.0	11	1937 ± 14.86	24.4
	2	10	663 ± 29.4	10	1904 ± 20.86	24.4

Table 28. Hours post-hatch (hph), tank, n, notochord and fork lengths, and oil and yolk areas from larvae imaged at CEI. No larvae survived after 56 hours post-hatch in Tank 1 and after 42 hours in Tank 2. N/A – measurements could not be taken.

Post hatch	*Tank*	*n*	*NL (µm)*	*TL (µm)*	*Oil area (mm²)*	*Yolk area (mm²)*
0 hour	1	1	3538	3597	0.15	0.74
	2	0	N/A	N/A	N/A	N/A
2 hours	1	0	N/A	N/A	N/A	N/A
	2	1	3197	3258	0.11	0.47
4 hours	1	0	N/A	N/A	N/A	N/A
	2	1	3552	3619	0.12	0.53
6 hours	1	1	3580	3600	0.12	0.40
	2	0	N/A	N/A	N/A	N/A
10 hours	1	1	4728	4829	0.14	0.57
	2	0	N/A	N/A	N/A	N/A
14 hours	1	1	4427	4495	0.17	0.47
	2	0	N/A	N/A	N/A	N/A
18 hours	1	1	5749	5798	0.15	0.46
	2	1	5458	5858	0.16	0.47
22 hours	1	1	6021	6100	0.16	0.41
	2	1	5873	6030	0.17	0.45
26 hours	1	0	N/A	N/A	N/A	N/A
	2	0	N/A	N/A	N/A	N/A
30 hours	1	1	6220	6414	0.18	0.26
	2	1	6573	6740	0.17	0.16
34 hours	1	0	N/A	N/A	N/A	N/A
	2	1	6652	6869	0.14	0.56
38 hours	1	1	6602	6888	0.12	0.14
	2	1	6882	7128	0.13	0.41
42 hours	1	0	N/A	N/A	N/A	N/A
	2	1	6364	6574	0.11	0.33
46 hours	1	1	6436	6571	0.12	0.17
52 hours	1	1	7311	7430	0.08	0.06
56 hours	1	1	6263	6494	0.16	0.07

Table 29. Hours post-hatch (hph), tank, n, notochord and fork lengths, and oil and yolk areas from fixed larvae in the lab. No larvae survived after 56 hours in Tank 1 and 42 hours in Tank 2. N/A – no larvae were fixed for that sampling period and tank.

Post hatch	*Tank*	*n*	*NL ± SD (μm)*	*TL ± SD (μm)*	*Oil area ± SD (mm²)*	*Yolk area ± SD (mm²)*
0 hour	1	2	3353 ± 6.866	3288 ± 22.20	0.17 ± 0.0083	0.81 ± 0.011
	2	16	3174 ± 59.70	3121 ± 64.74	0.14 ± 0.014	0.72 ± 0.022
2 hours	1	24	3655 ± 56.29	3594 ± 52.07	0.18 ± 0.012	0.73 ± 0.024
	2	27	3689 ± 82.91	3618 ± 83.63	0.19 ± 0.011	0.78 ± 0.029
4 hours	1	24	3496 ± 211.3	3435 ± 220.8	0.16 ± 0.034	0.59 ± 0.060
	2	50	3794 ± 195.4	3725 ± 186.3	0.18 ± 0.014	0.66 ± 0.041
6 hours	1	1	4144	4048572	0.16	0.72
	2	29	4186 ± 182.8	4077 ± 174.1	0.20 ± 0.019	0.71 ± 0.037
10 hours	1	1	4361	4240	0.21	0.68
	2	8	4611 ± 207.0	4490 ± 209.2	0.21 ± 0.015	0.69 ± 0.024
14 hours	1	6	4884 ± 189.9	4789 ± 191.4	0.20 ± 0.013	0.63 ± 0.063
	2	3	4923 ± 161.2	4828 ± 131.2	0.21 ± 0.0072	0.63 ± 0.014
18 hours	1	2	5351 ± 33.85	5204 ± 3.993	0.20 ± 0.021	0.59 ± 0.0071
	2	2	5049 ± 73.16	4928 ± 78.36	0.19 ± 0.026	0.57 ± 0.066
22 hours	1	3	5422 ± 152.1	5303 ± 182.3	0.19 ± 0.013	0.48 ± 0.031
	2	1	5653	5484	0.20	0.67
26 hours	1	0	N/A	N/A	N/A	N/A
	2	5	5182 ± 388.0	5043 ± 357.4	0.19 ± 0.0099	0.46 ± 0.044
30 hours	1	2	5755 ± 208.2	5589 ± 182.4	0.18 ± 0.0035	0.35 ± 0.014
	2	0	N/A	N/A	N/A	N/A
34 hours	1	0	N/A	N/A	N/A	N/A
	2	5	5216 ± 577.5	5062 ± 601.9	0.16 ± 0.019	0.32 ± 0.071
38 hours	1	3	5454 ± 673.3	5342 ± 617.5	0.16 ± 0.13	0.33 ± 0.057
	2	1	6376	6194	0.16	0.46
42 hours	1	5	4123 ± 361.0	3972 ± 380.9	0.12 ± 0.014	0.32 ± 0.042
	2	2	5871 ± 107.8	5662 ± 65.09	0.15 ± 0.015	0.44 ± 0.019
46 hours	1	1	5044	4902	0.12	0.24
56 hours	1	1	6554	6508	0.11	0.49

Appendix B: Figures

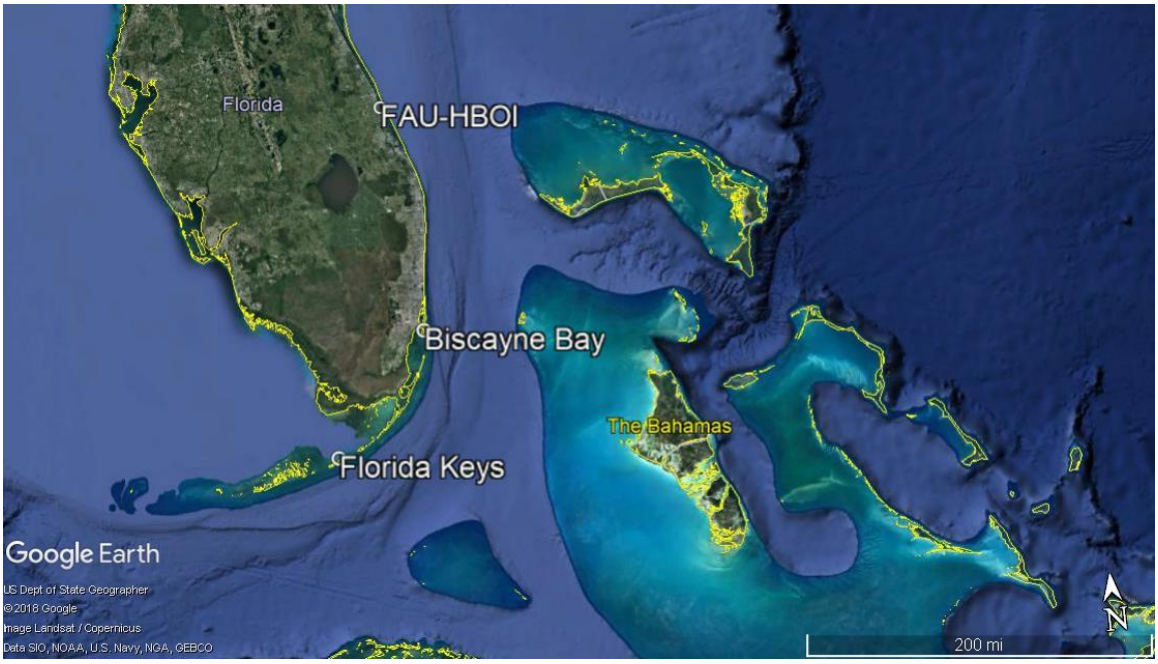

Figure 1. Location of FAU-HBOI facilities and general Bonefish collection locations in South Florida

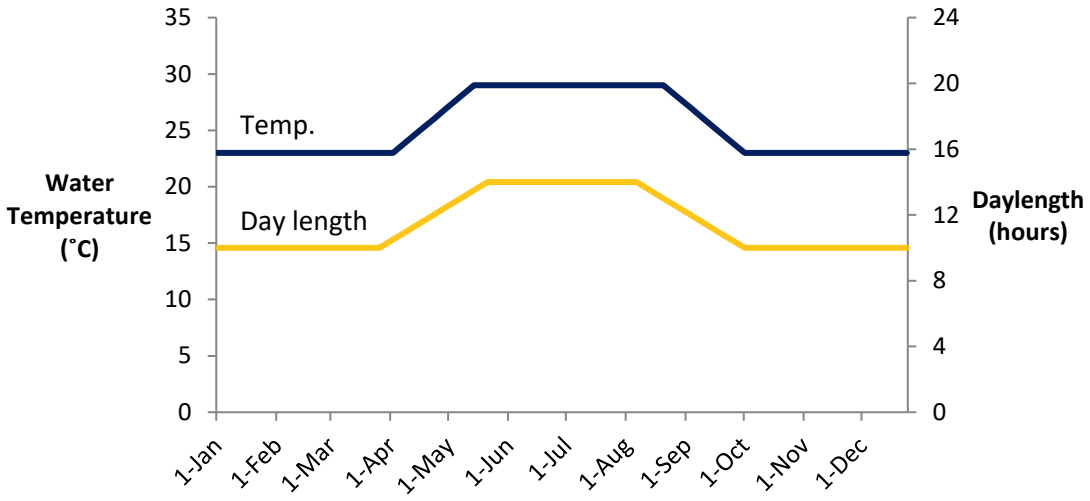

Figure 2. Temperature and photoperiod in the natural cycle for conditioning Bonefish. This cycle was used twice for Bonefish from the Florida Keys and once for Bonefish from Biscayne Bay.

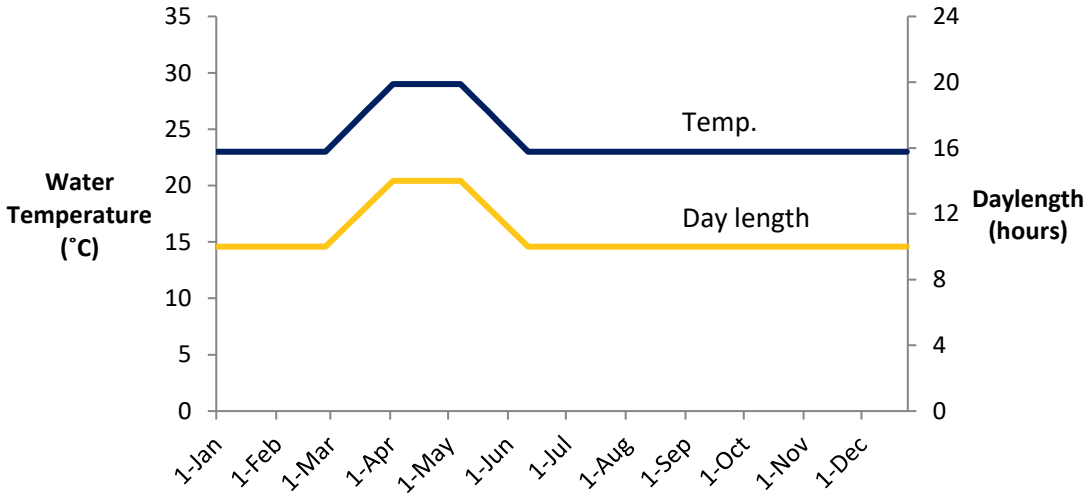

Figure 3. Temperature and photoperiod in the short cycle for conditioning Bonefish. This cycle was used for the third spawning attempt with the Florida Keys Bonefish.

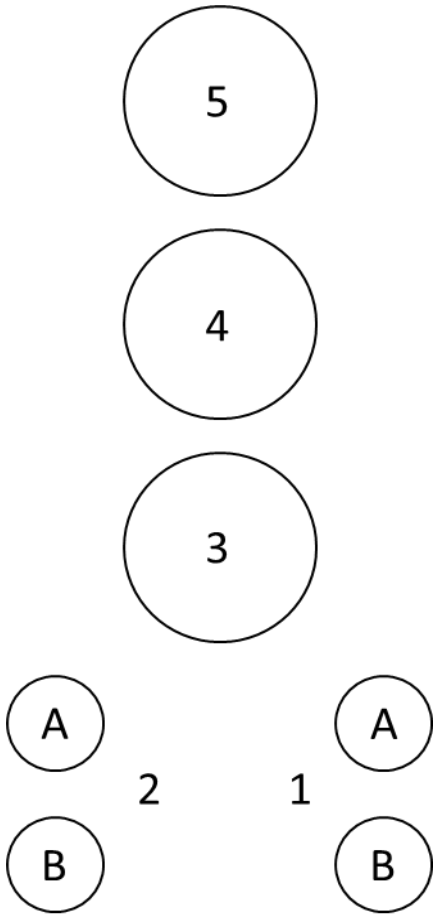

Figure 4. Layout of recirculating tanks used for conditioning and spawning trials at FAU-HBOI. Systems 1 and 2 consist of two tanks each (A & B), which share sumps. Tank 3 was not used in this study.

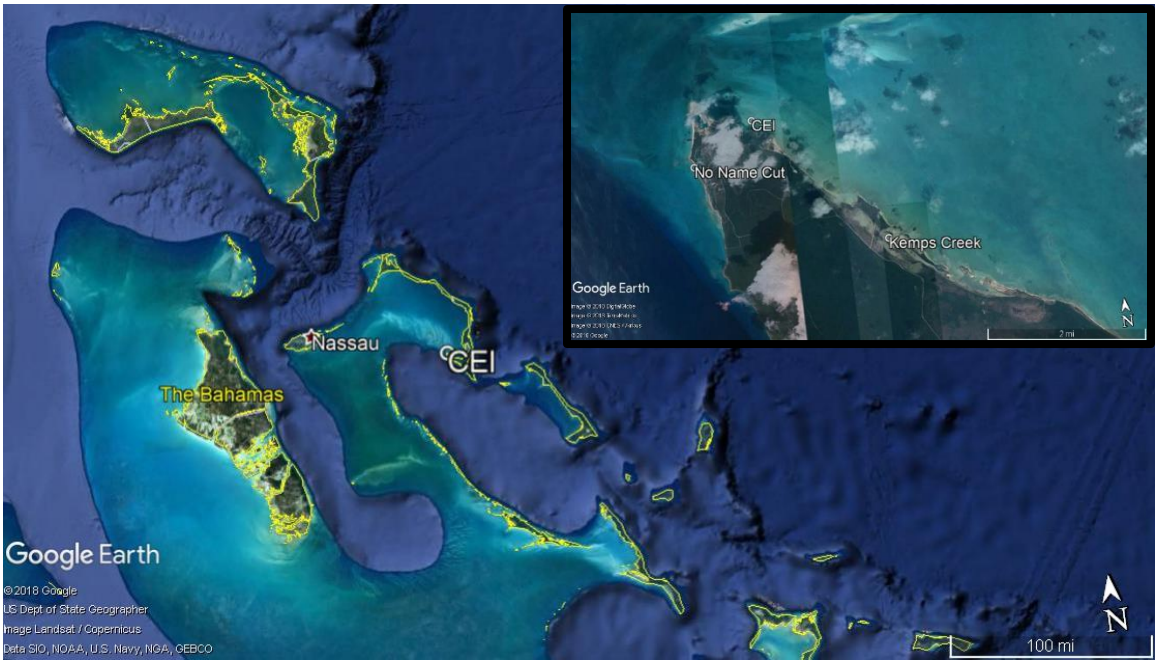

Figure 5. Eleuthera, The Bahamas. Inset: Cape Eleuthera and the three collection sites.

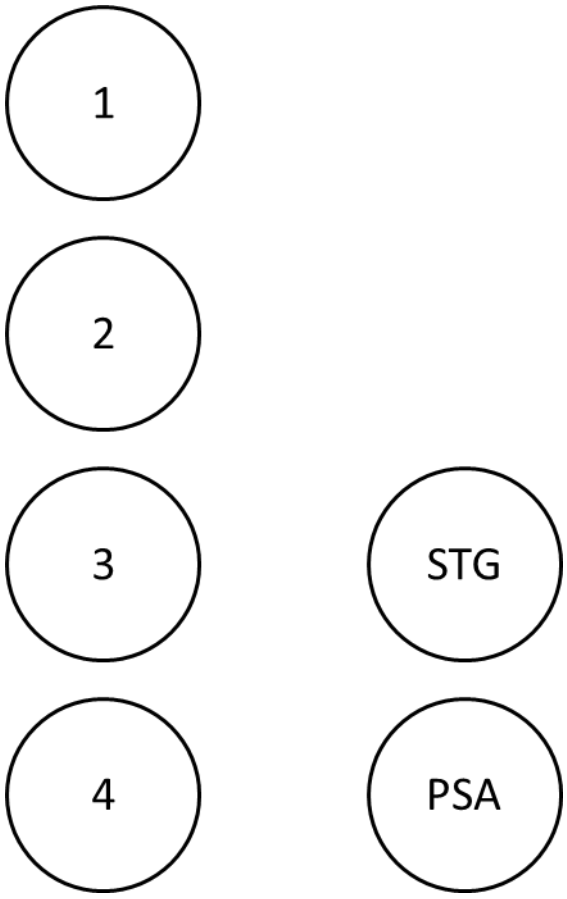

Figure 6. Layout of tanks used for conditioning and spawning trials at CEI. Tanks 1-4 were used for the long-term group. STG – short-term group, PSA – pre-spawn aggregation group. All six tanks were on flow-through water and under a pavilion with ambient light.

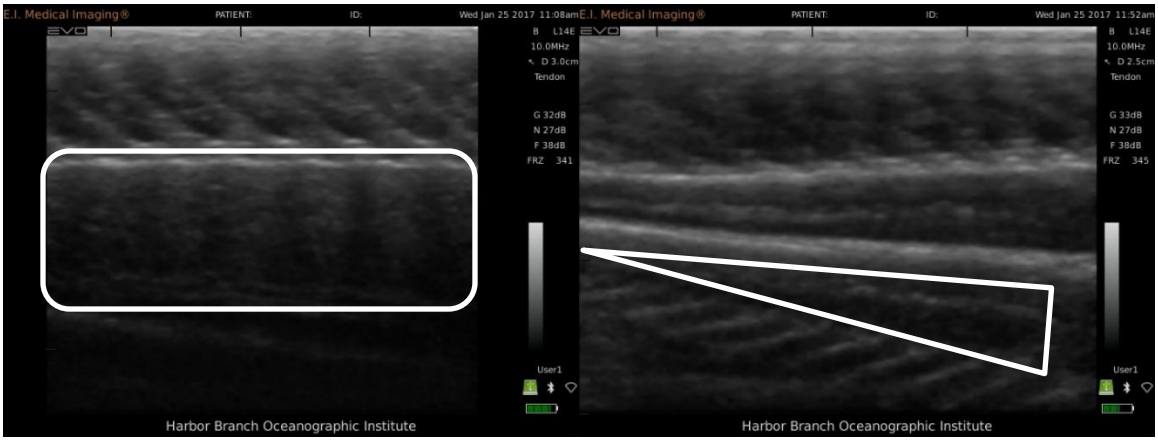

Figure 7. Sonograms of female (517, left) and male (596, right) gonads. Ovaries (white box) alternate between hypo- (darker) and hyperechoic (lighter) on the sonogram. Testes are hypoechoic and run along the intestines (white triangle), which are hyperechoic.

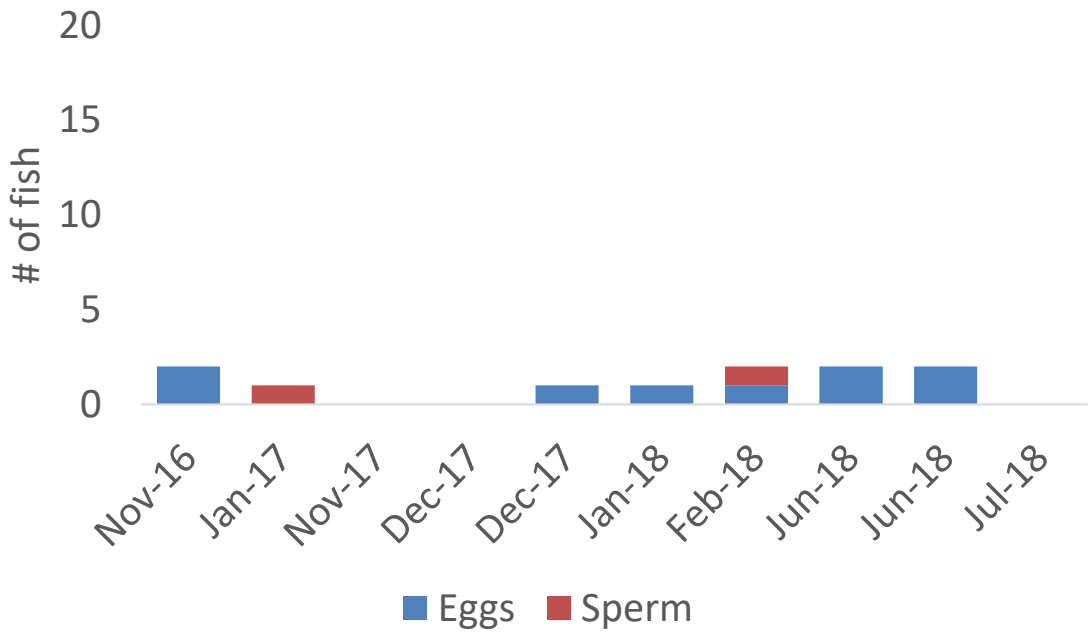

Figure 8. Gametes collected from Bonefish in the Florida Keys group during this study.

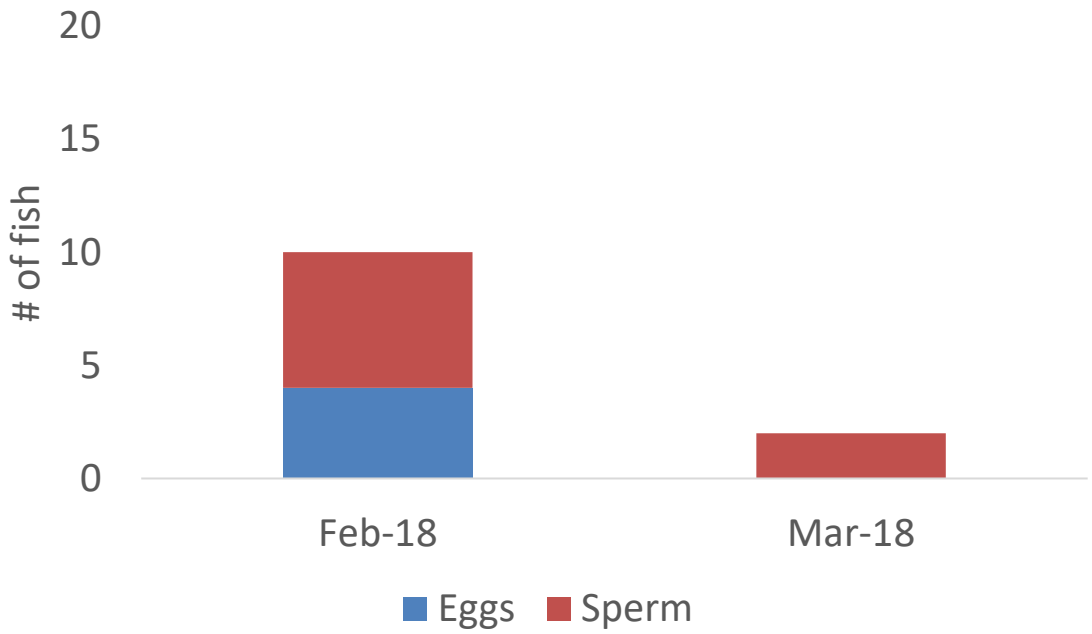

Figure 9. Gametes collected from Bonefish in the Biscayne Bay group during this study.

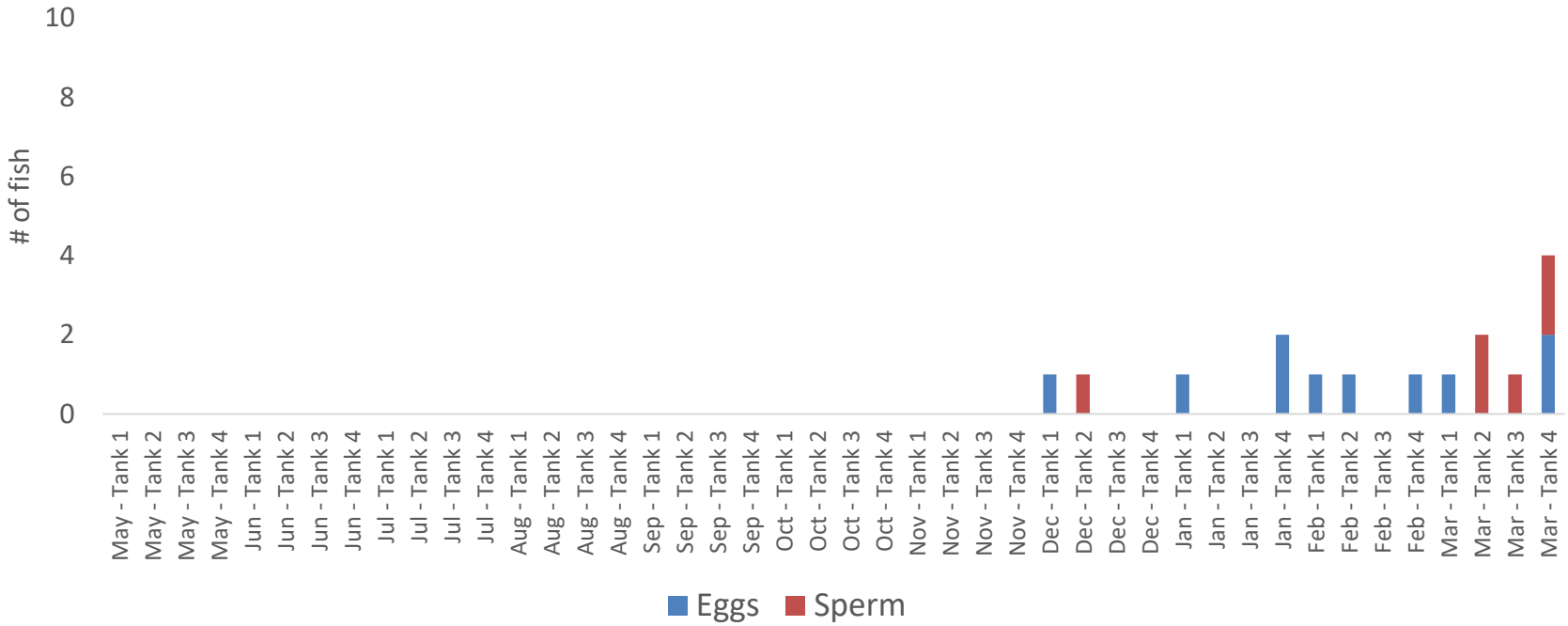

Figure 10. Gametes collected from Bonefish at CEI for each tank in the long-term group from May 2017 to March 2018.

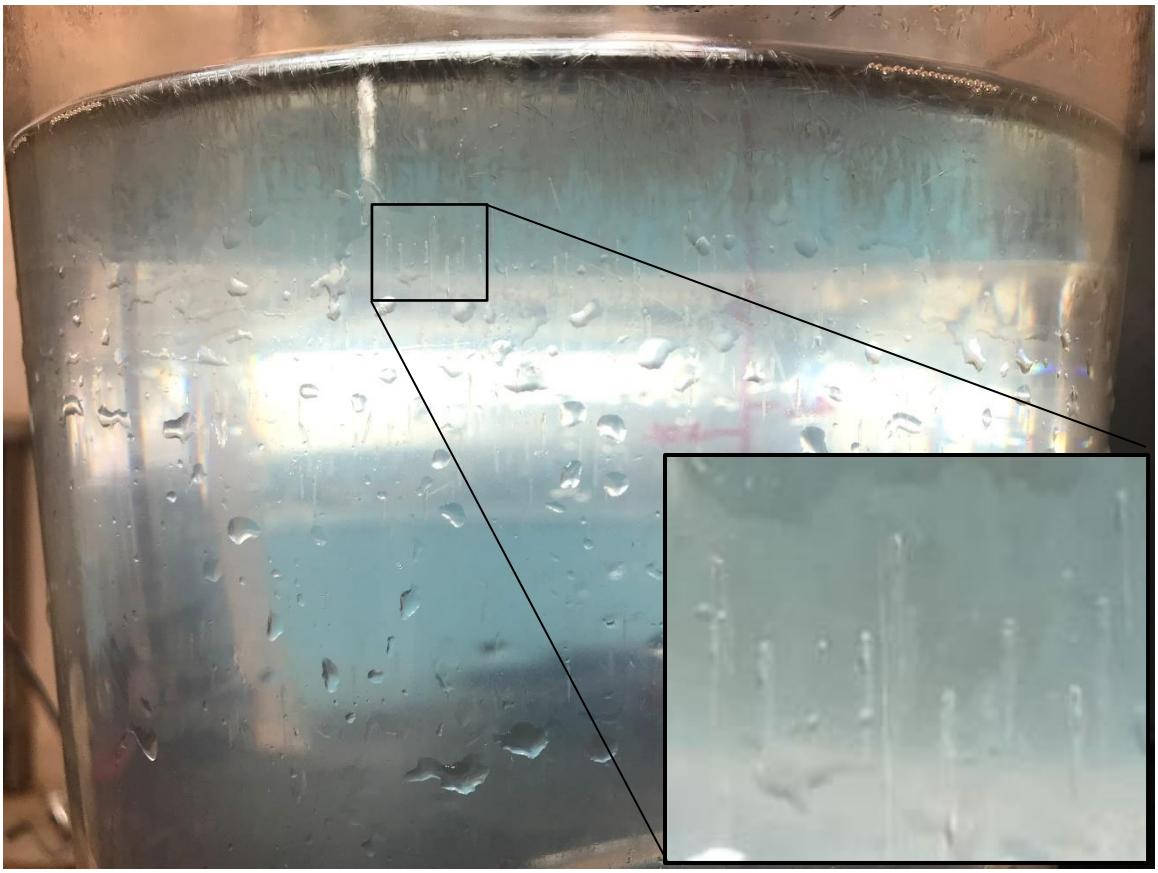

Figure 11. Six liter polycarbonate bucket used for egg incubation and larval rearing. This image is of larvae 4 hph. Larvae oriented vertically (inset) and congregated near the surface, but were also observed throughout the water column (not pictured).

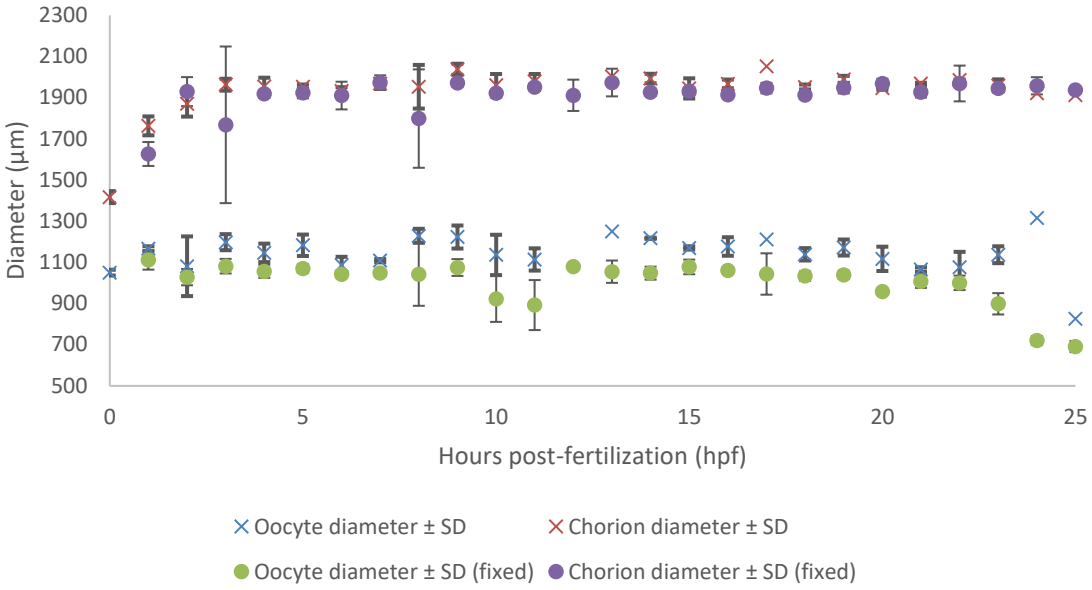

Figure 12. Mean oocyte and chorion diameters of eggs from Tank 1 imaged (crosses and bold error bars) and fixed (circles) at CEI. Measurements of imaged eggs could not be taken at 12 hpf. N=1 for 13, 17, 24, and 25 hpf for both series.

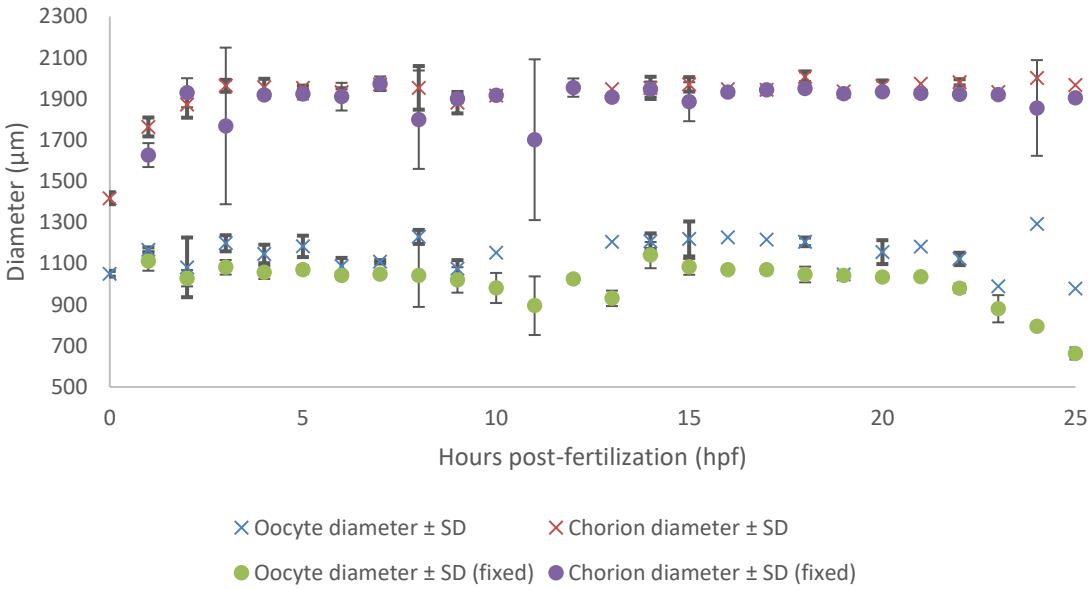

Figure 13. Mean oocyte and chorion diameters of eggs from Tank 2 imaged (crosses and bold error bars) and fixed (circles) at CEI. Measurements of imaged eggs could not be taken at 11 or 12 hpf. N=1 for 10, 13, 16, 17, 19, 21, 23, 24, and 25 hpf for both series.

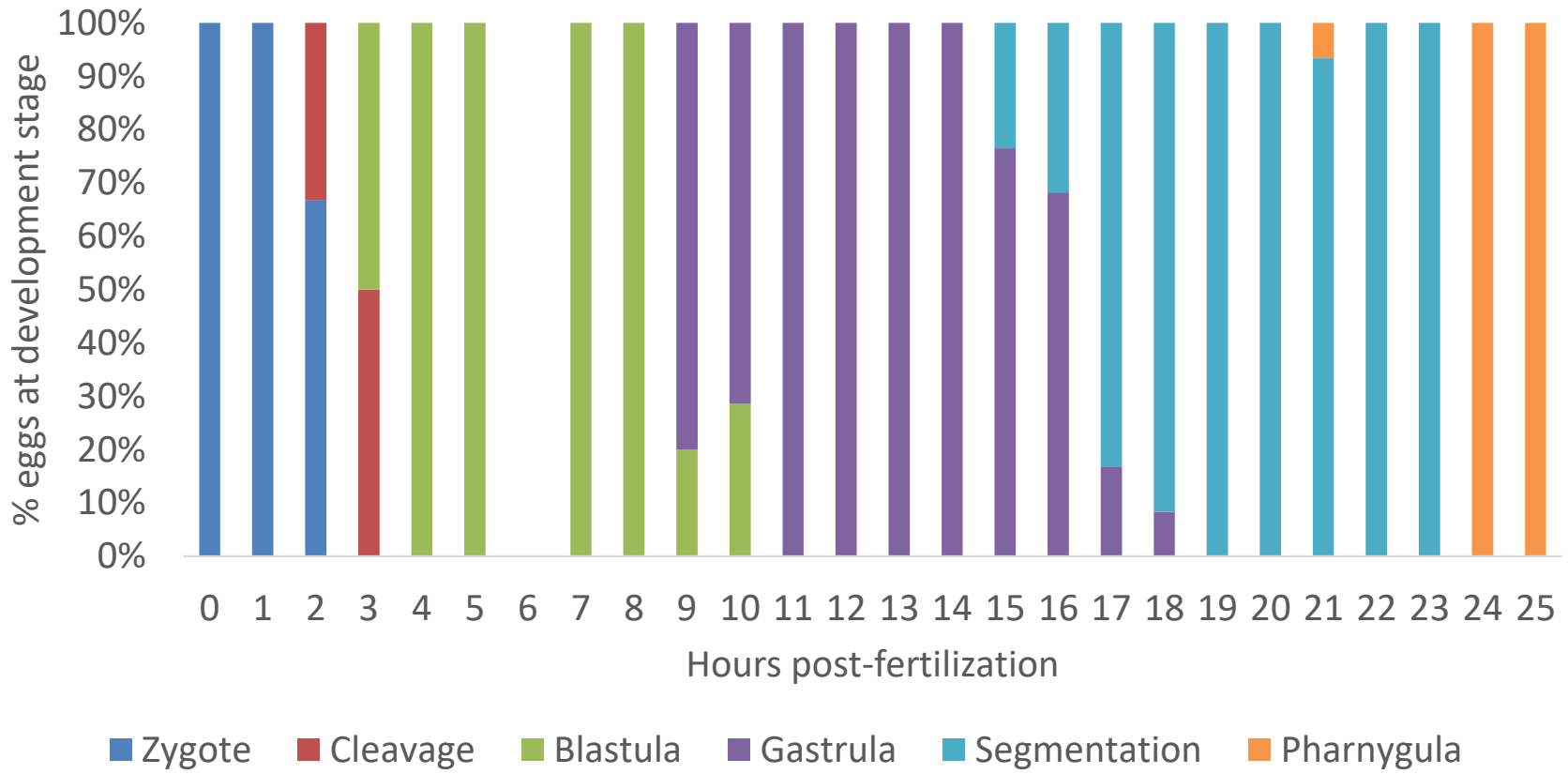

Figure 14. Percent of fresh eggs observed at each developmental stage for each hour post-fertilization (hpf). No stages could be determined at 6 hpf.

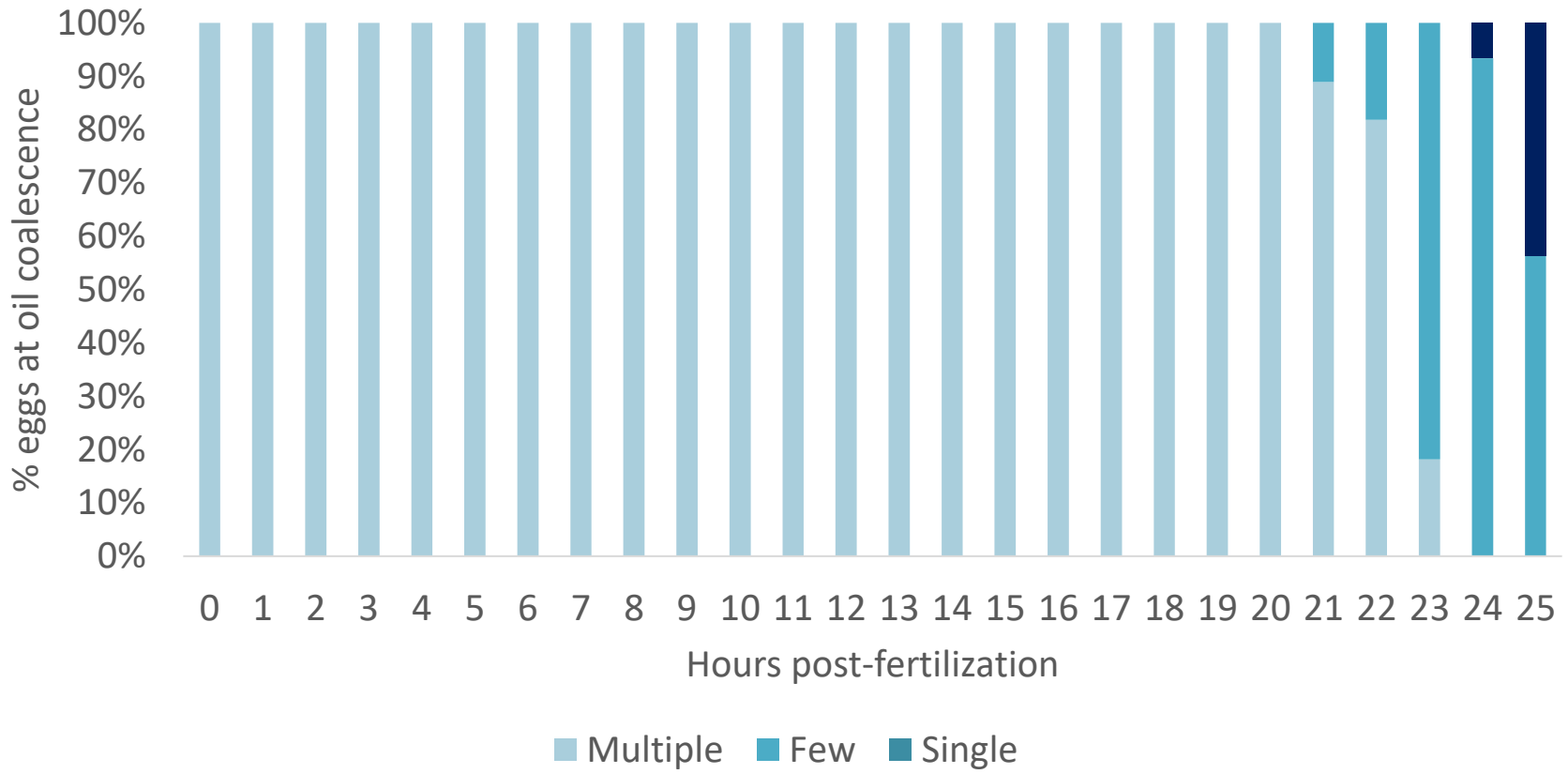

Figure 15. Percent of fresh eggs at each oil droplet coalescence stage for each hour post-fertilization. Multiple=7+ droplets, Few=2-6 droplets, Single=1 droplet.

Figure 16. Image of eggs at CEI from Tank 1 at 1 hpf. Eggs are in the zygote stage. The white egg with three oil droplets was considered dead. Image taken using darkfield lighting. Scale bar = 0.5 mm.

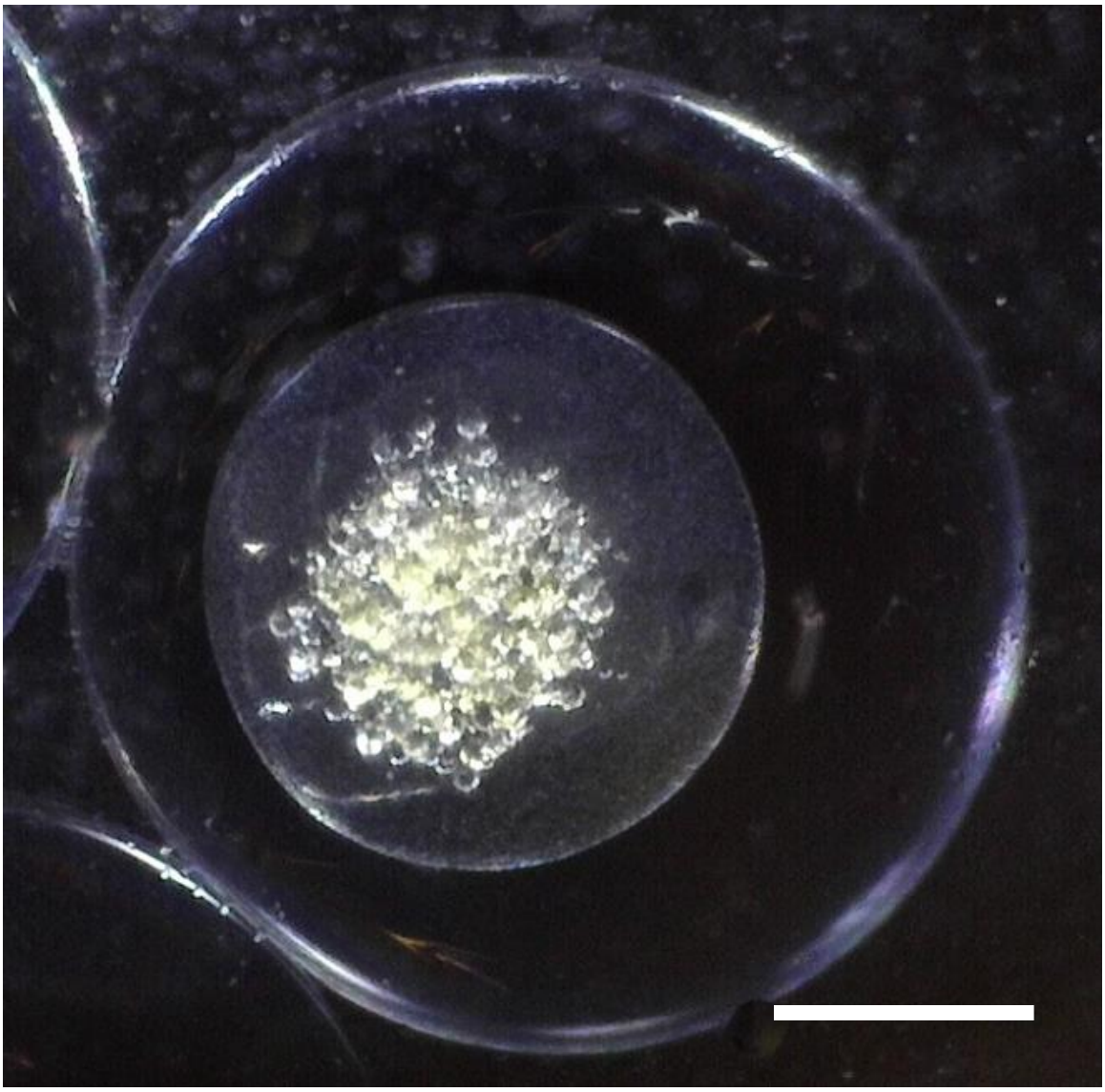

Figure 17. Image of an egg at CEI from Tank 1 at 2 hpf. The egg is in the cleavage stage (16 cells). Image taken using darkfield lighting. Scale bar = 0.5 mm.

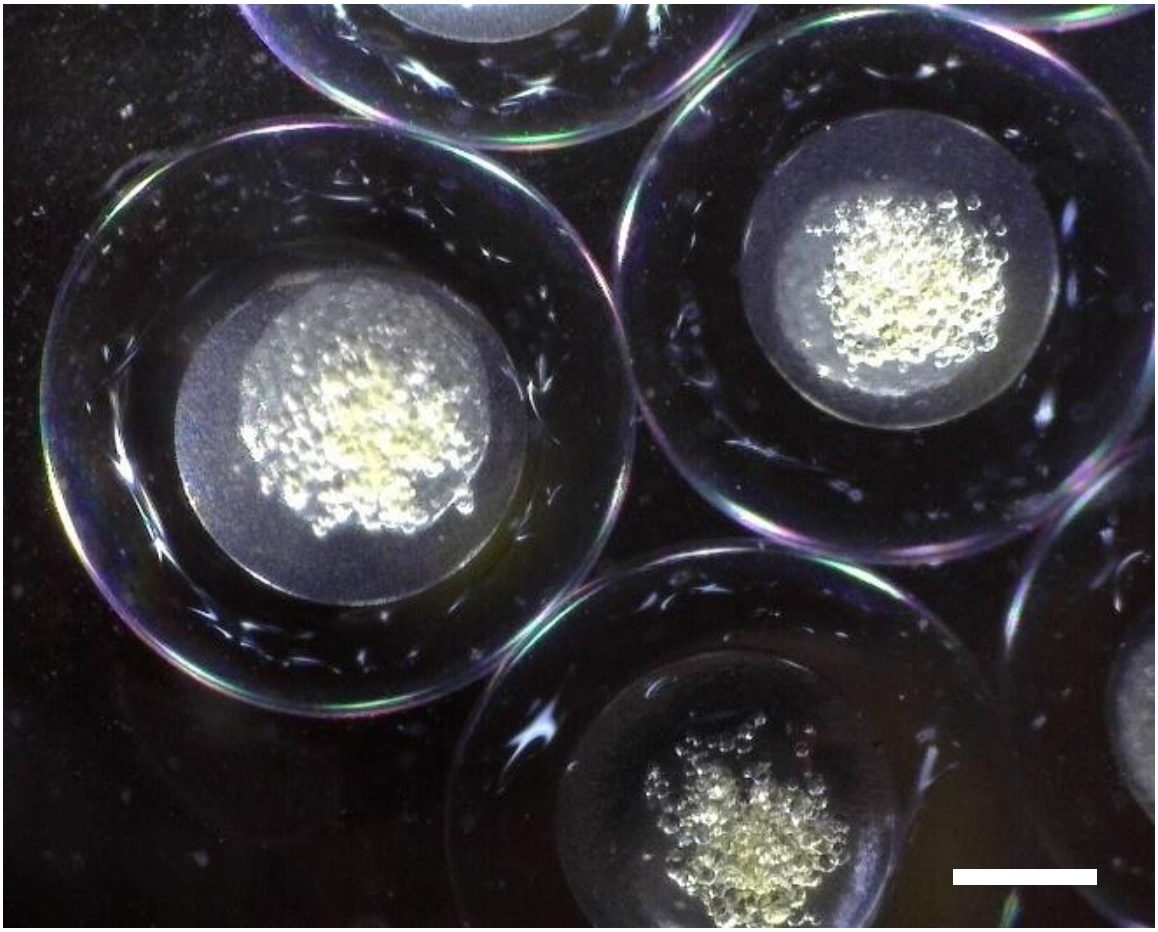

Figure 18. Image of eggs at CEI from Tank 1 at 4 hpf. Eggs are in the blastula stage. Image taken using darkfield lighting. Scale bar = 0.5 mm.

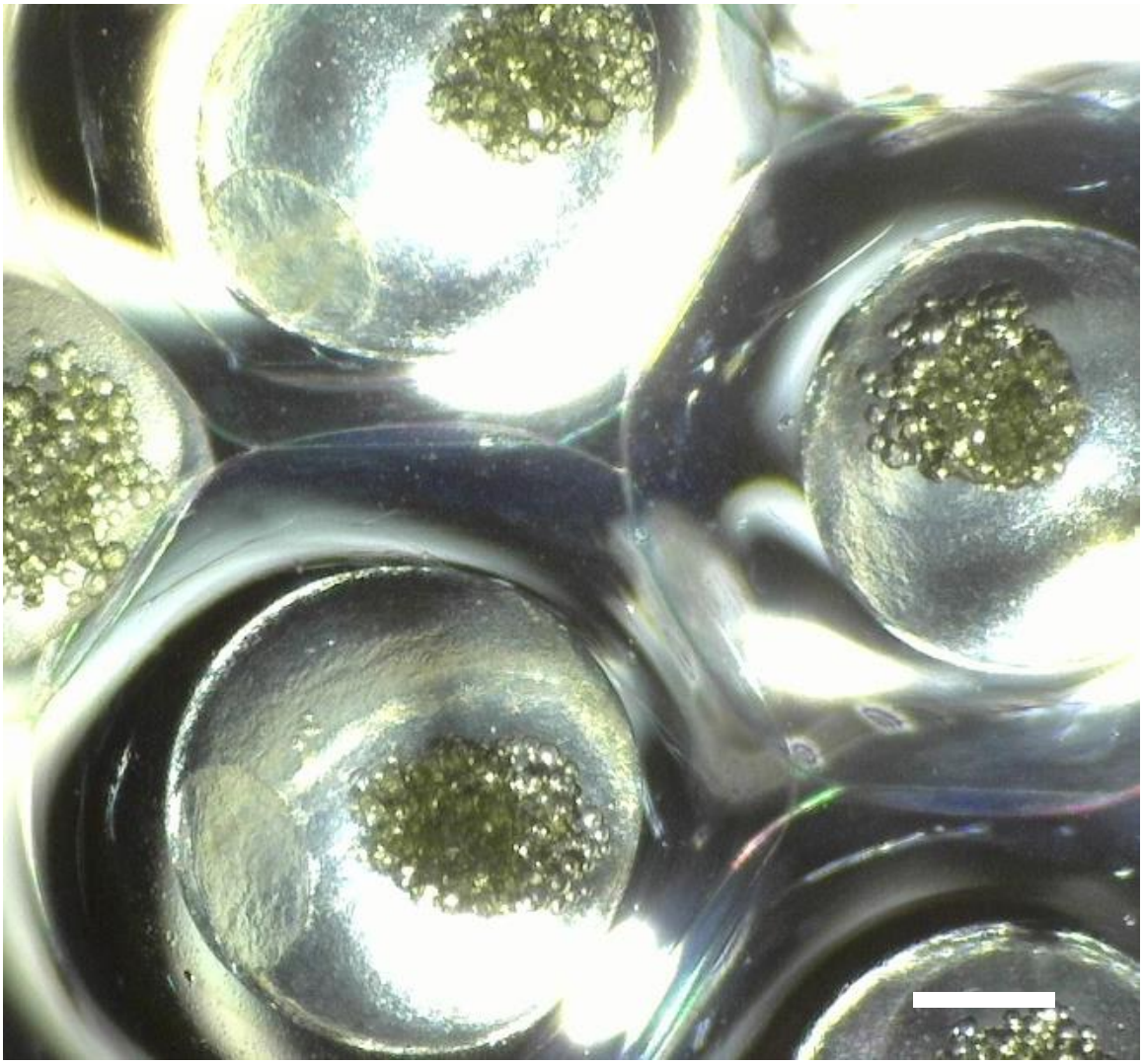

Figure 19. Image of eggs taken at CEI from Tank 2 at 11 hpf. Eggs are in gastrula stage. Image taken using light field lighting. Scale bar = 0.5 mm.

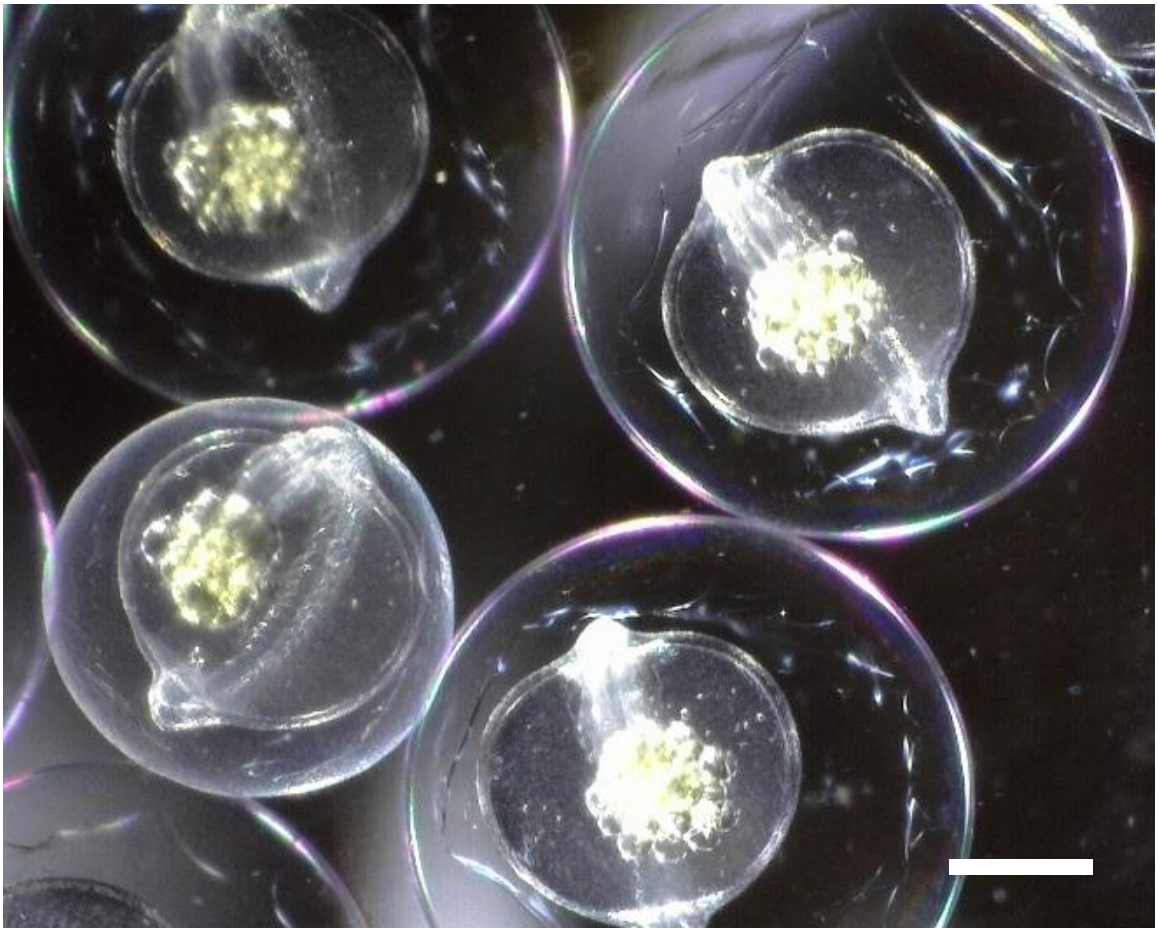

Figure 20. Image of eggs at CEI from Tank 2 at 19 hpf. Eggs are in the segmentation stage. Image taken using darkfield lighting. Scale bar = 0.5 mm.

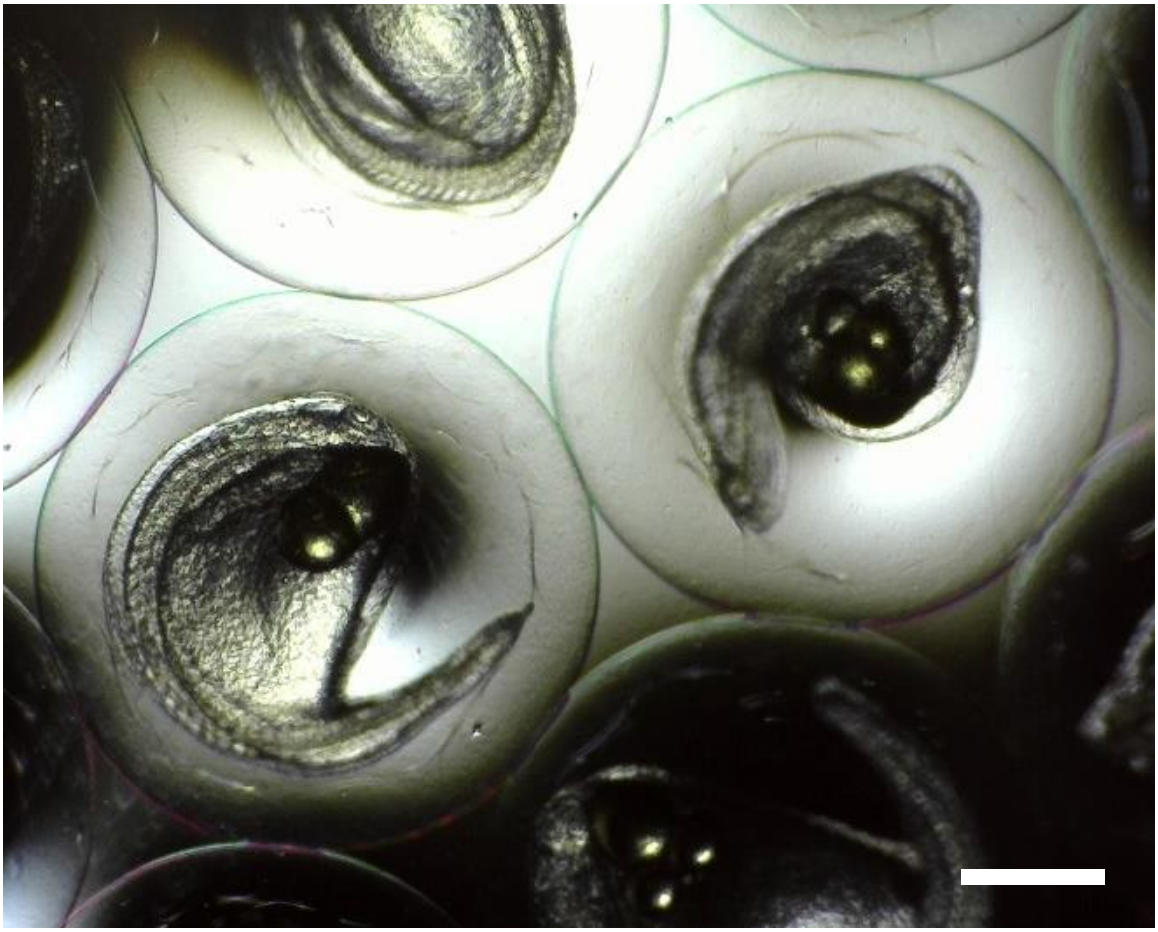

Figure 21. Image of eggs at CEI from Tank 1 at 24 hpf, 2 hours before hatching. Eggs are in the pharyngula stage. Oil droplets are nearing total coalescence. Image taken using darkfield lighting. Scale bar = 0.5 mm.

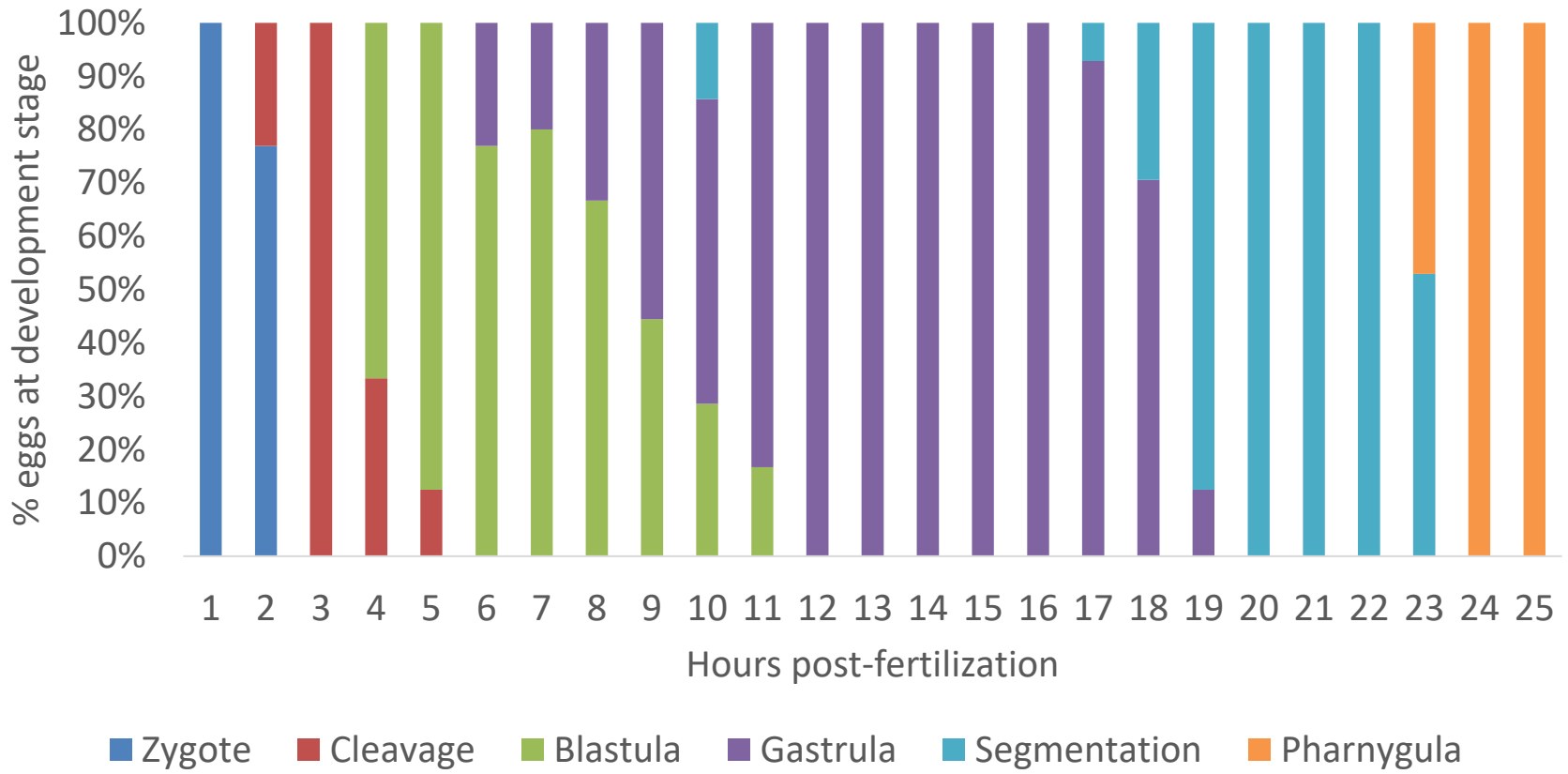

Figure 22. Percent of fixed eggs observed at each developmental stage for each hour post-fertilization (hpf).

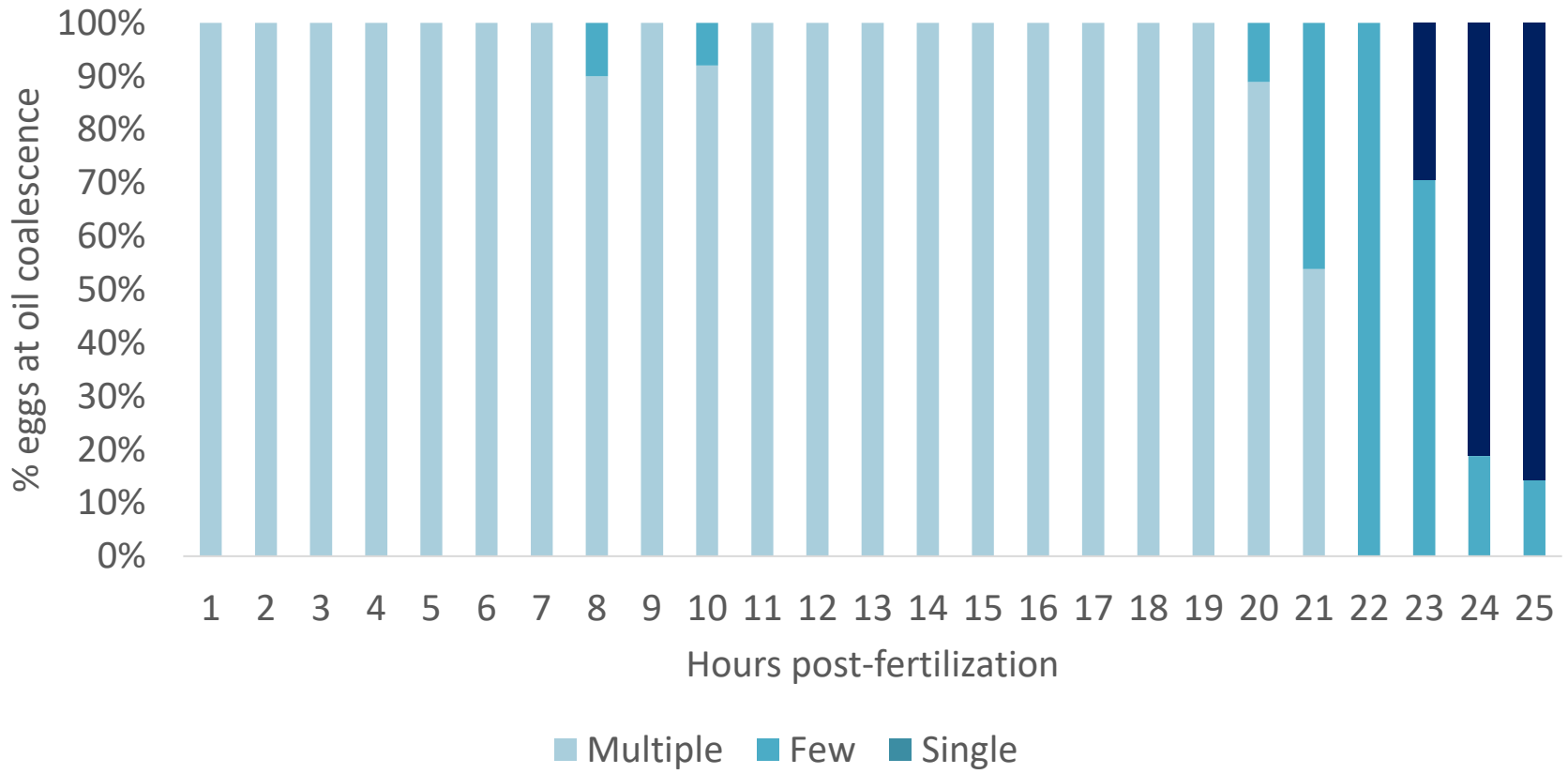

Figure 23. Percent of fixed eggs at each oil droplet coalescence stage for each hour post-fertilization. Multiple=7+ droplets, Few=2-6 droplets, Single=1 droplet.

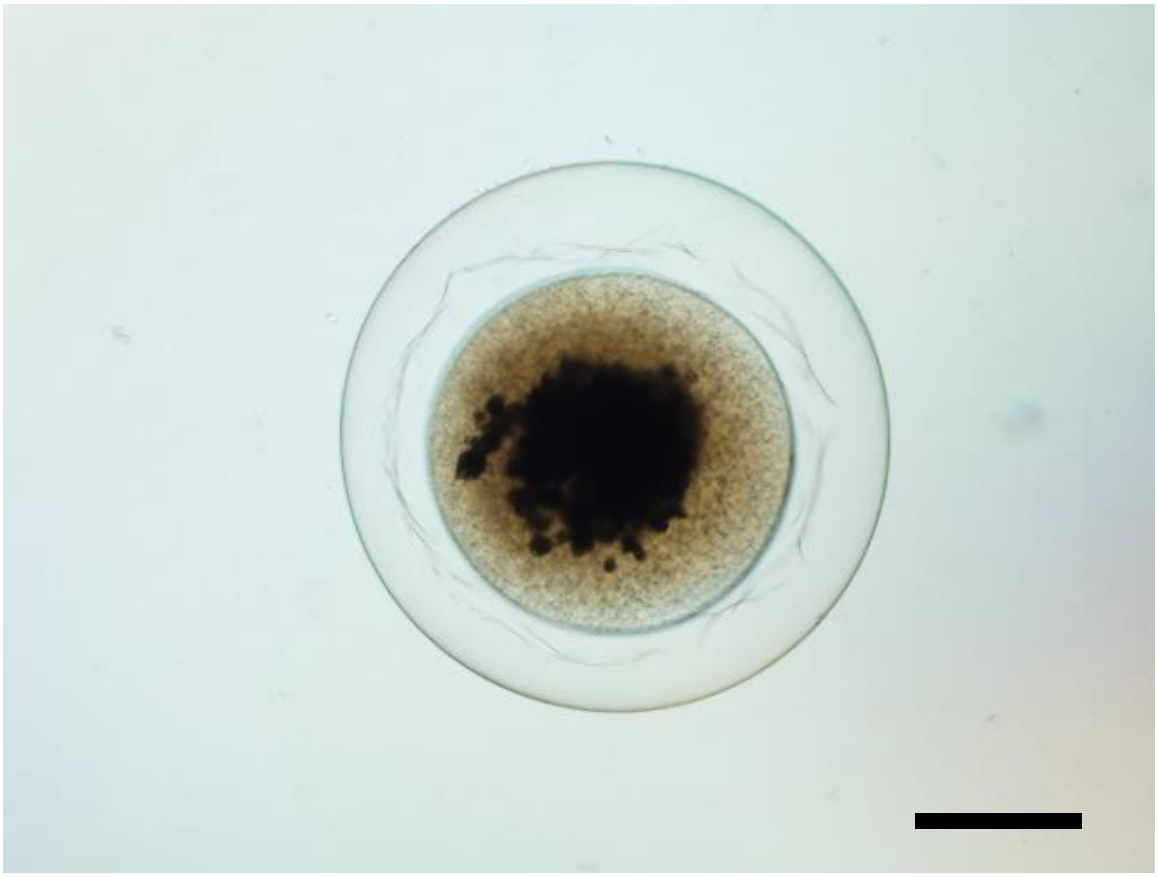

Figure 24. Image of a fixed egg from Tank 1 at 1 hpf. The egg is in the zygote stage. Scale bar = 0.5 mm.

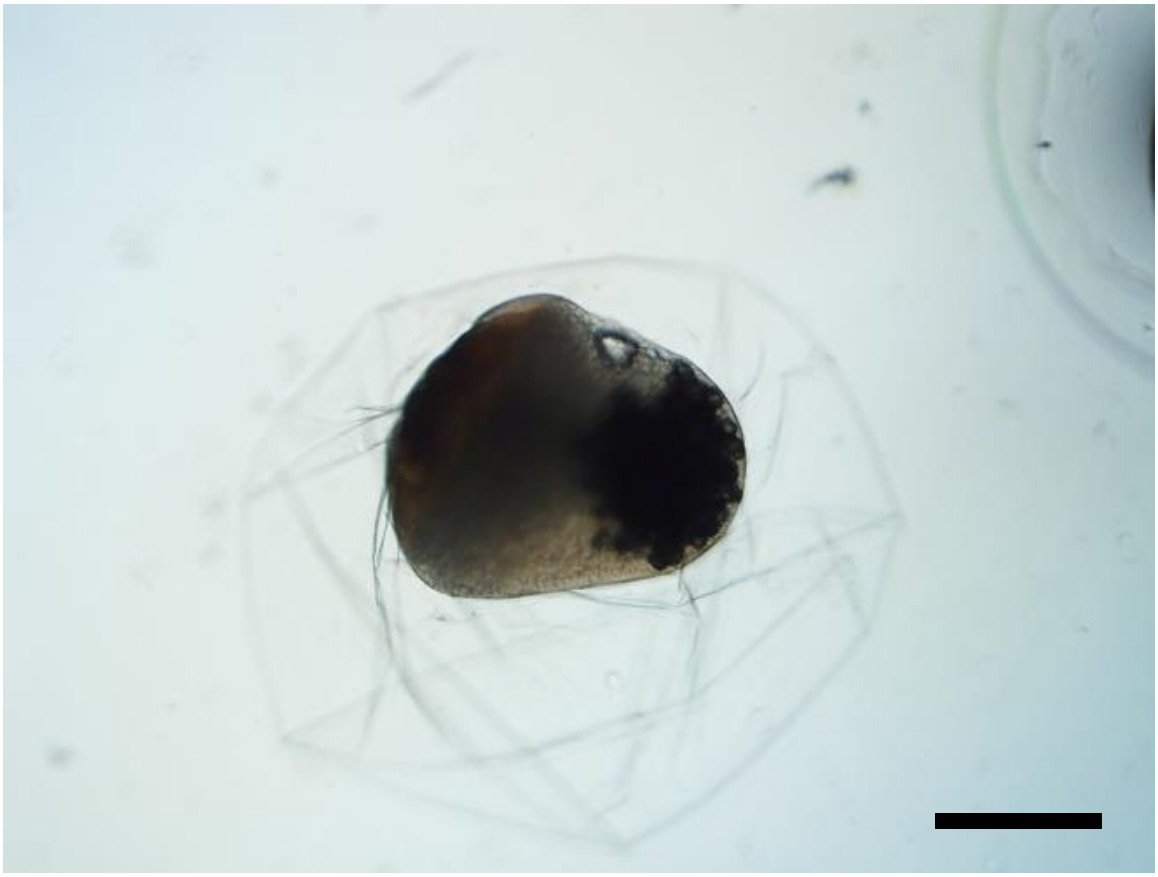

Figure 25. Image of a fixed egg from Tank 1 at 2 hpf. The egg is in the cleavage stage. The chorion has been damaged. Scale bar = 0.5 mm.

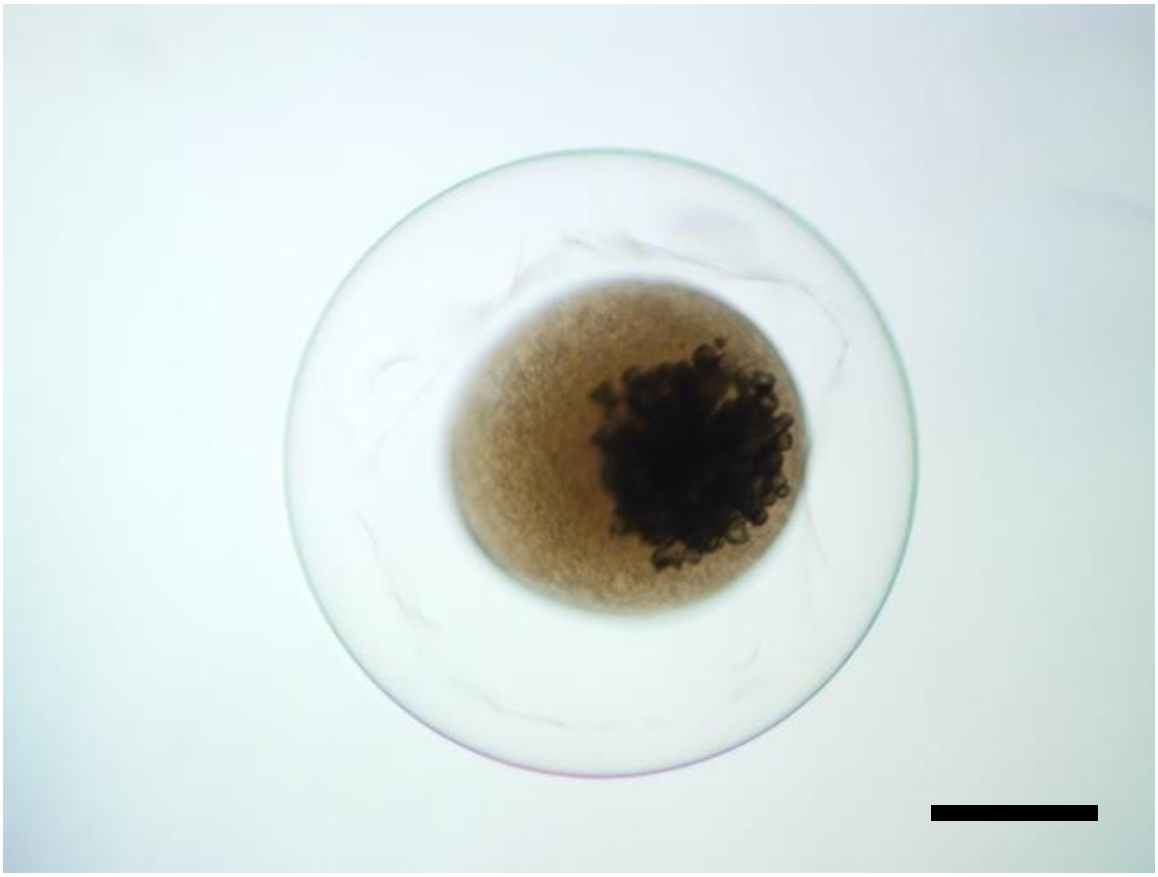

Figure 26. Image of a fixed egg from Tank 1 at 5 hpf. The egg is in the blastula stage. Scale bar = 0.5 mm.

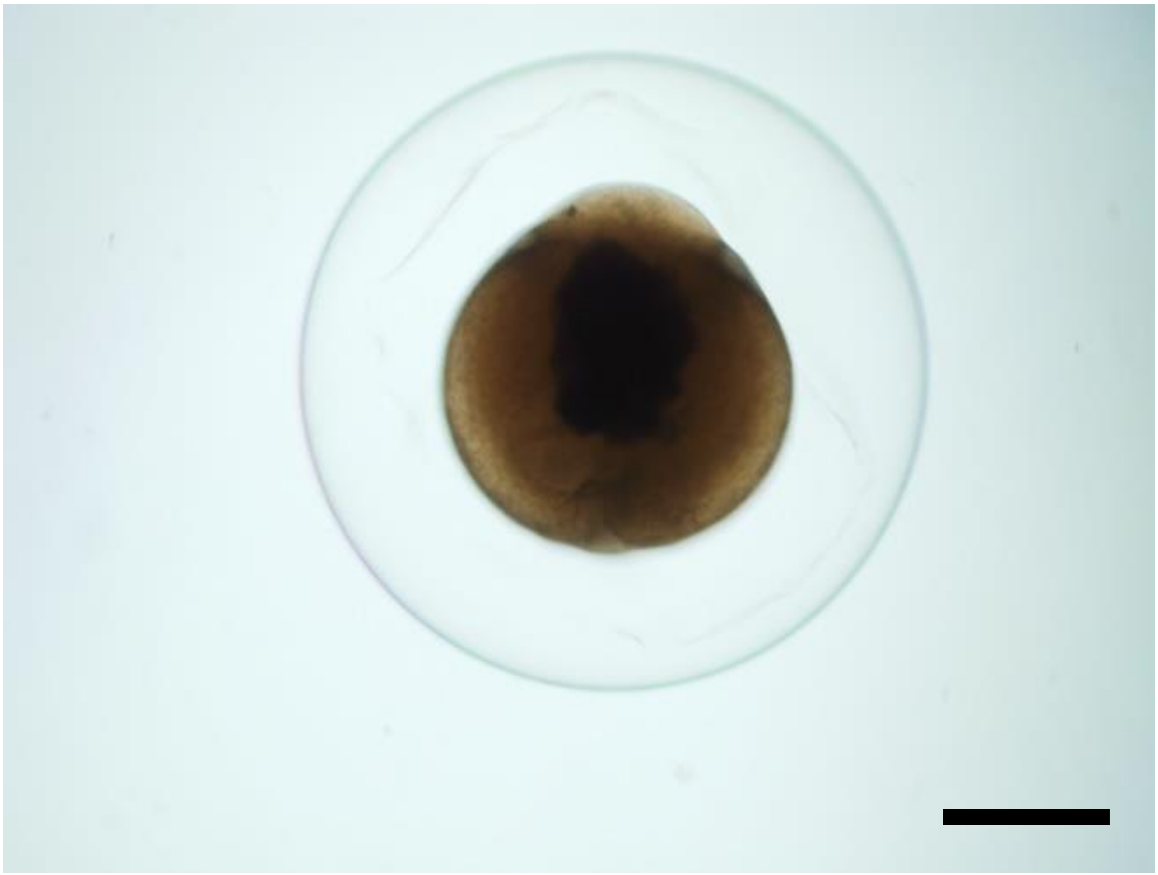

Figure 27. Image of a fixed egg from Tank 2 at 12 hpf. The egg is in the gastrula stage. Scale bar = 0.5 mm.

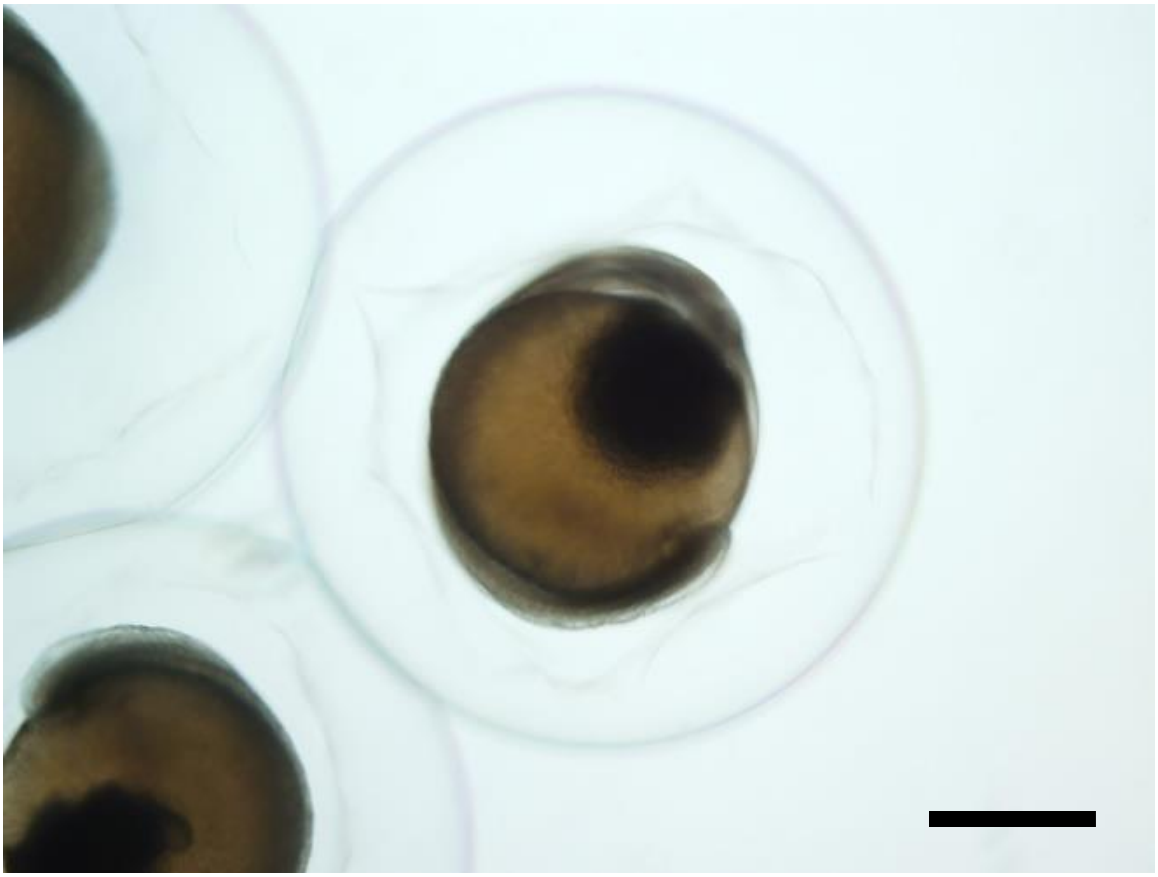

Figure 28. Image of a fixed egg from Tank 1 at 20 hpf. The egg is in the segmentation stage. Scale bar = 0.5 mm.

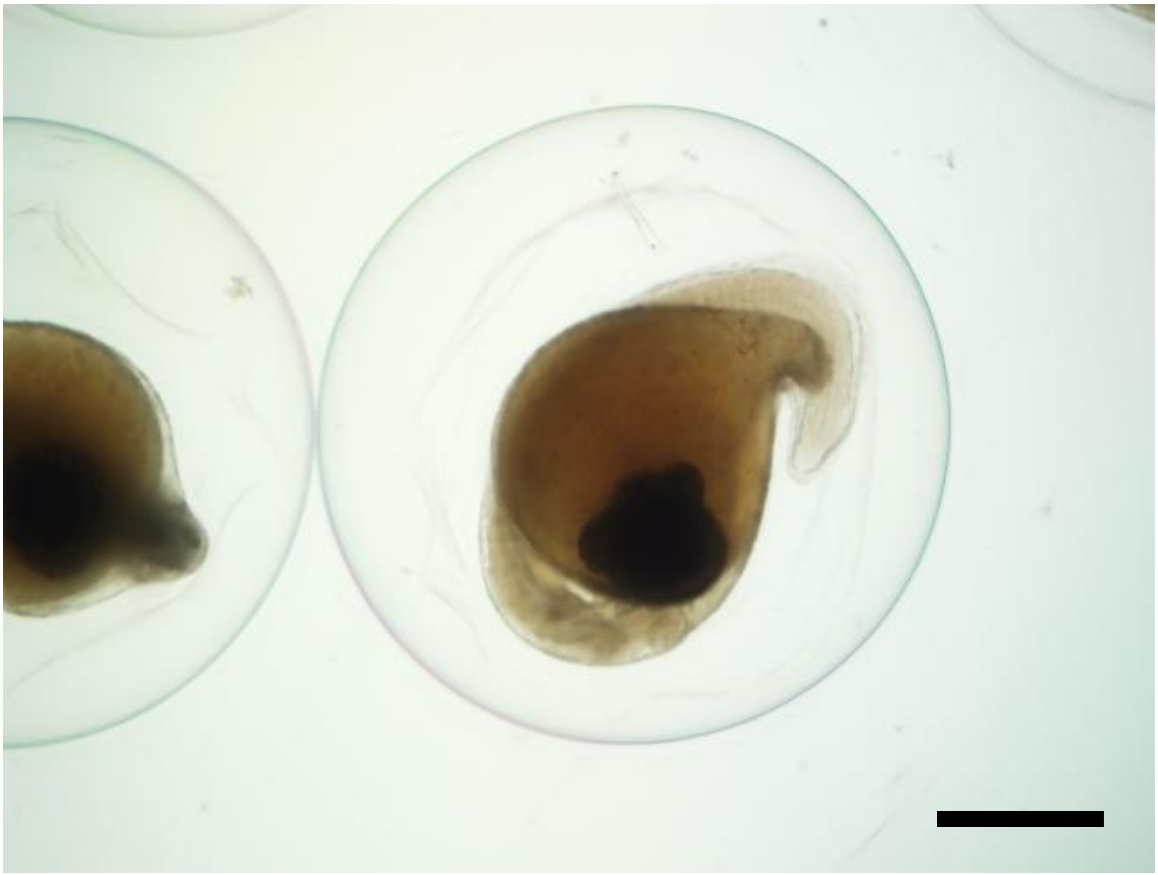

Figure 29. Image of a fixed egg from Tank 1 at 23 hpf, 3 hours before hatching. The egg is in the pharyngula stage. Oil droplets are nearing total coalescence. Scale bar = 0.5 mm.

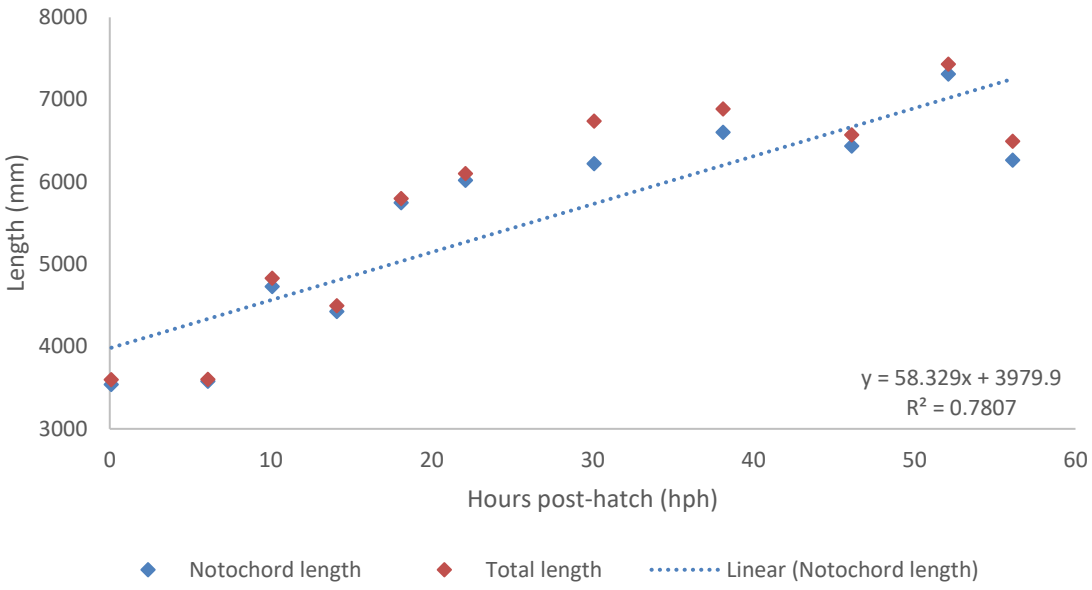

Figure 30. Notochord and total lengths of larvae imaged from Tank 1 at CEI. N=1 for all measurements. No larvae survived after 56 hph. Measurements could not be taken at 1, 2, 4, 26, 34, and 42 hpf. Linear regression is for notochord length and P<0.001, df(10).

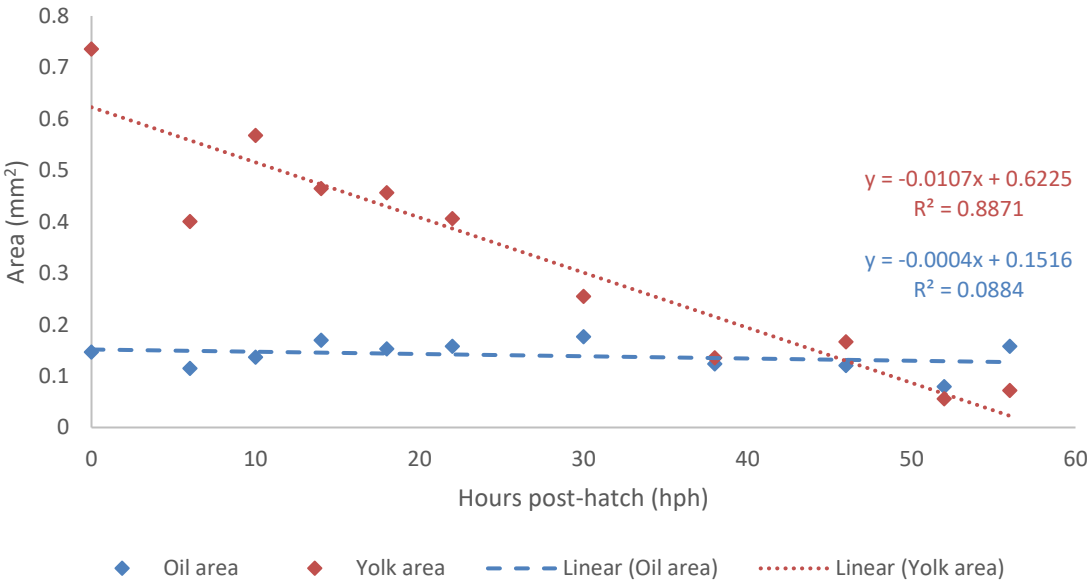

Figure 31. Oil and yolk areas of larvae imaged from Tank 1 at CEI. No larvae survived after 56 hph. N=1 for all measurements. Measurements could not be taken at 1, 2, 4, 26, 34, and 42 hpf. P<0.001 for the linear regression of yolk area, df(9), and P=0.41 for oil area, df(9).

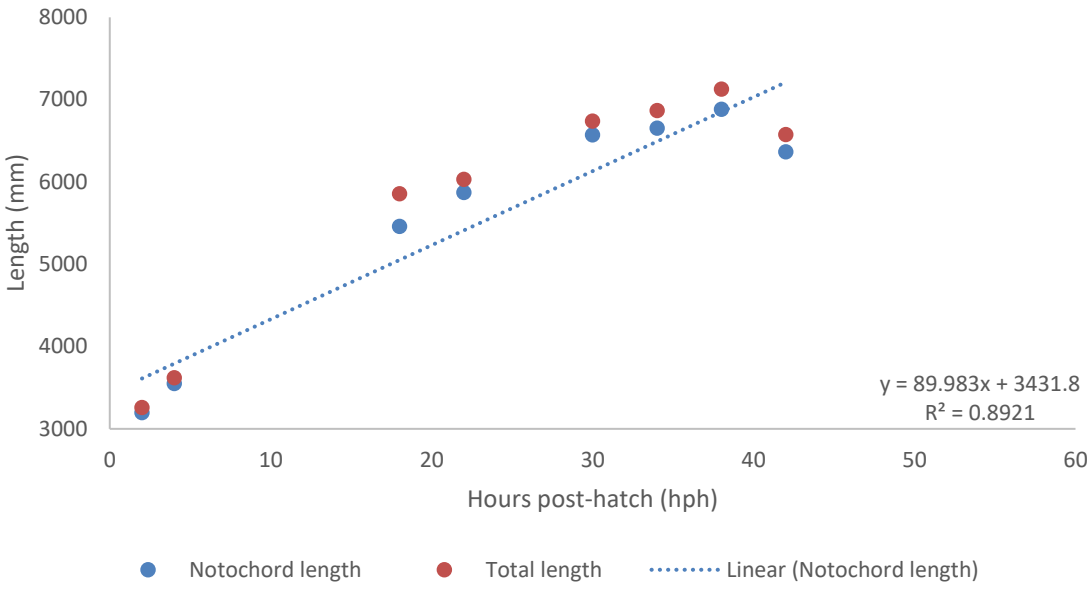

Figure 32. Notochord and total lengths of larvae imaged from Tank 2 at CEI. No larvae survived after 42 hph. N=1 for all measurements. Measurements could not be taken at 0, 1, 6, 10, 14, and 26 hph. Linear regression is for notochord length and P<0.01, df(6).

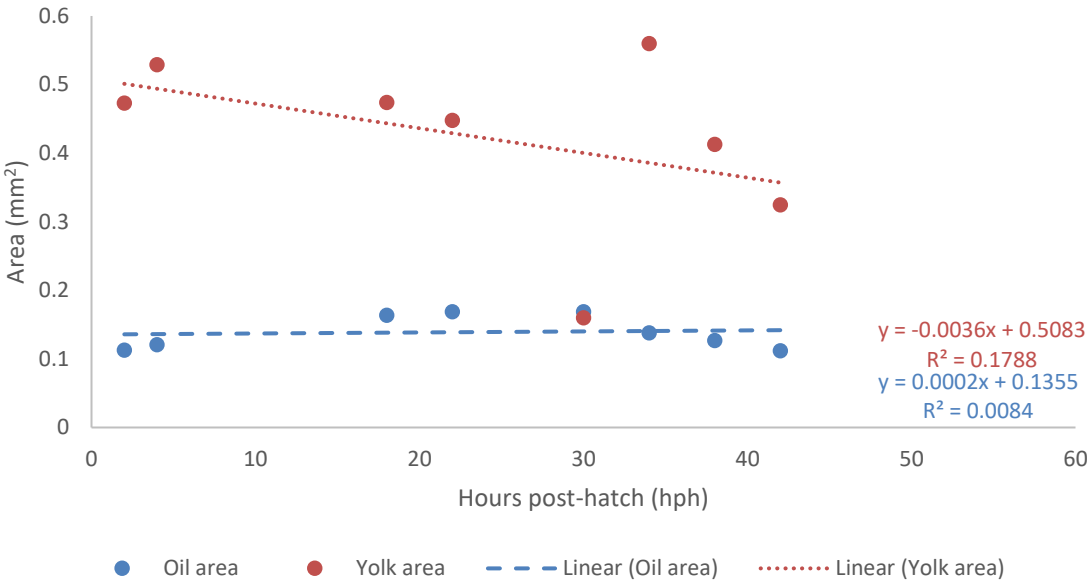

Figure 33. Oil and yolk areas of larvae imaged from Tank 2 at CEI. No larvae survived after 42 hph. N=1 for all measurements. Measurements could not be taken at 0, 1, 6, 10, 14, and 26 hph. P=0.36 for the linear regression of yolk area, df(6), and P=0.64 for oil area, df(6).

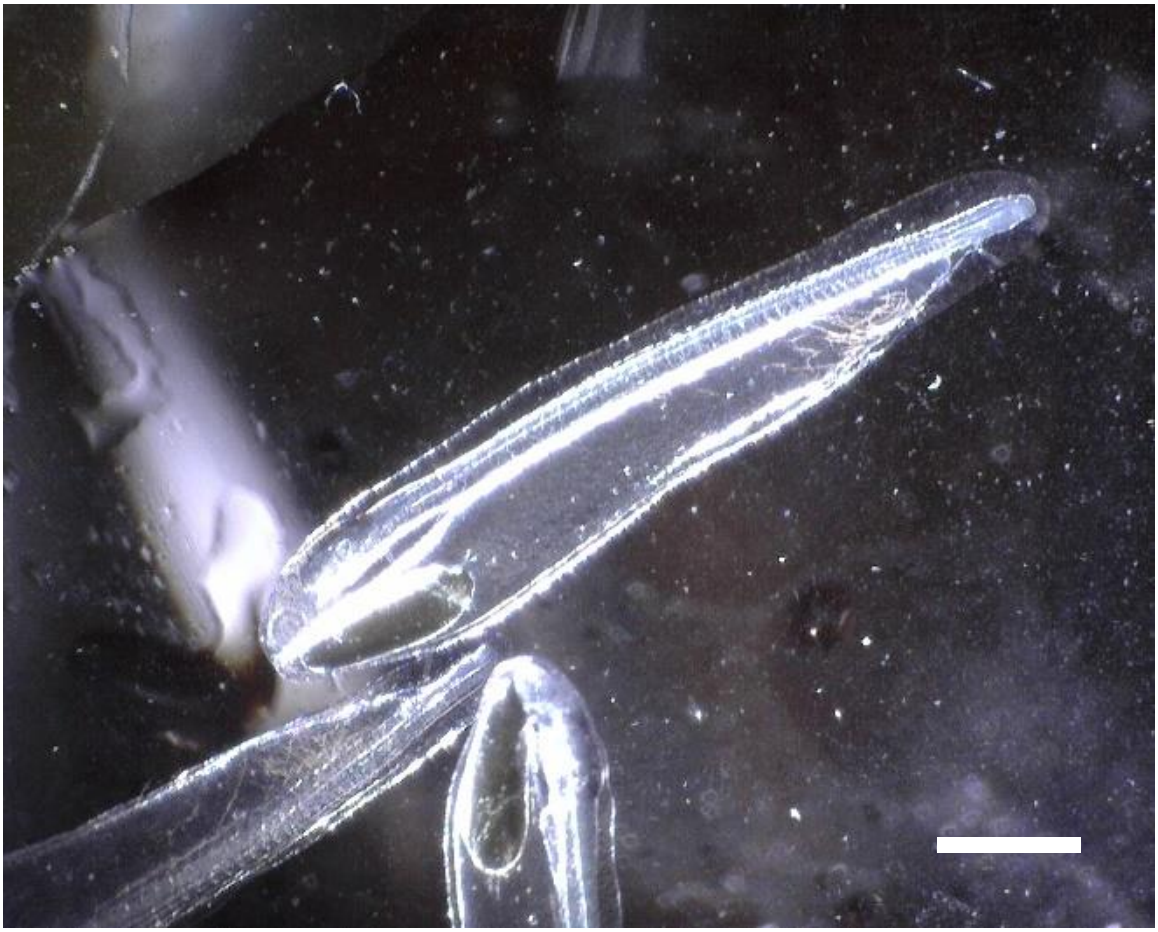

Figure 34. Larvae imaged at CEI from Tank 2 at 0 hph. Oil droplets were completely coalesced at hatching. Image taken using darkfield lighting. Scale bar = 0.5 mm.

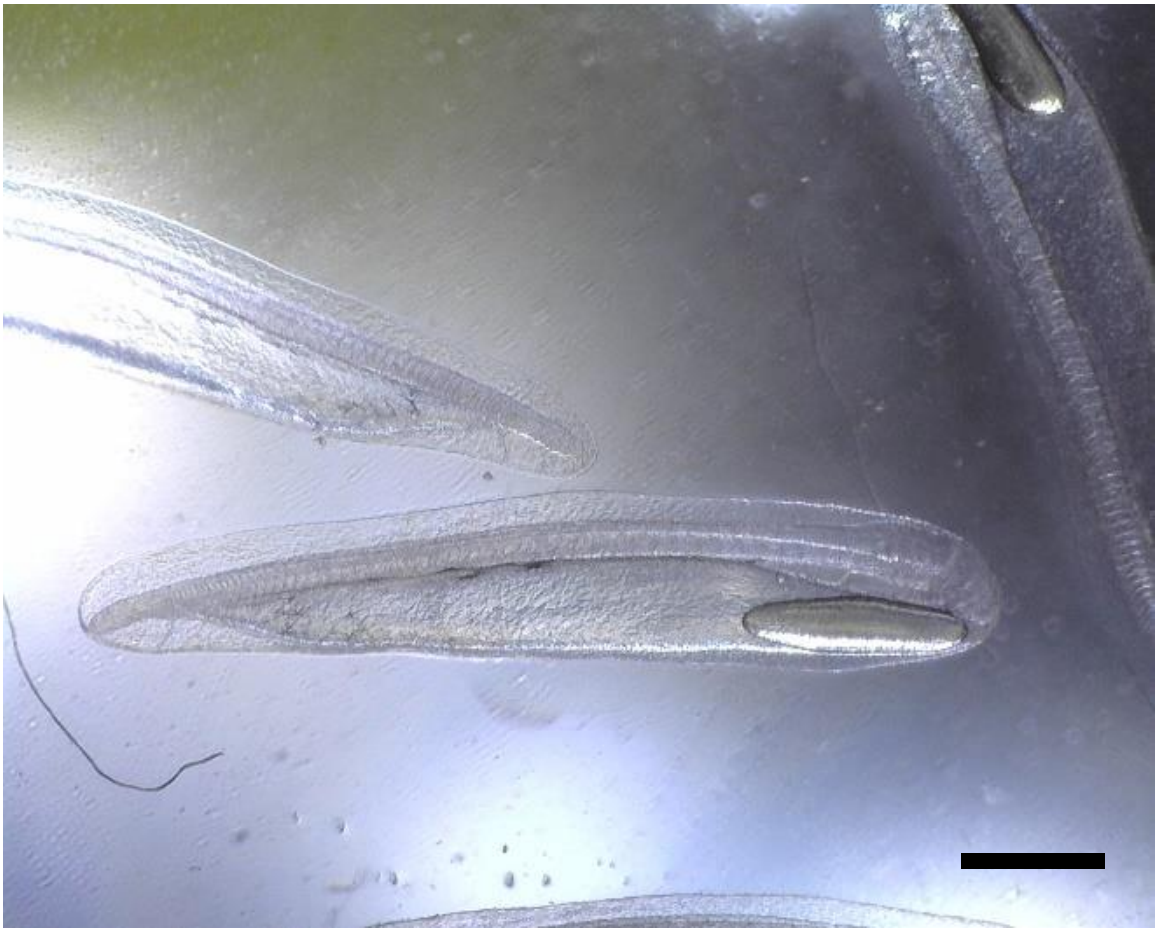

Figure 35. Larvae imaged at CEI from Tank 1 at 2 hph. Image taken using light field lighting. Scale bar = 0.5 mm.

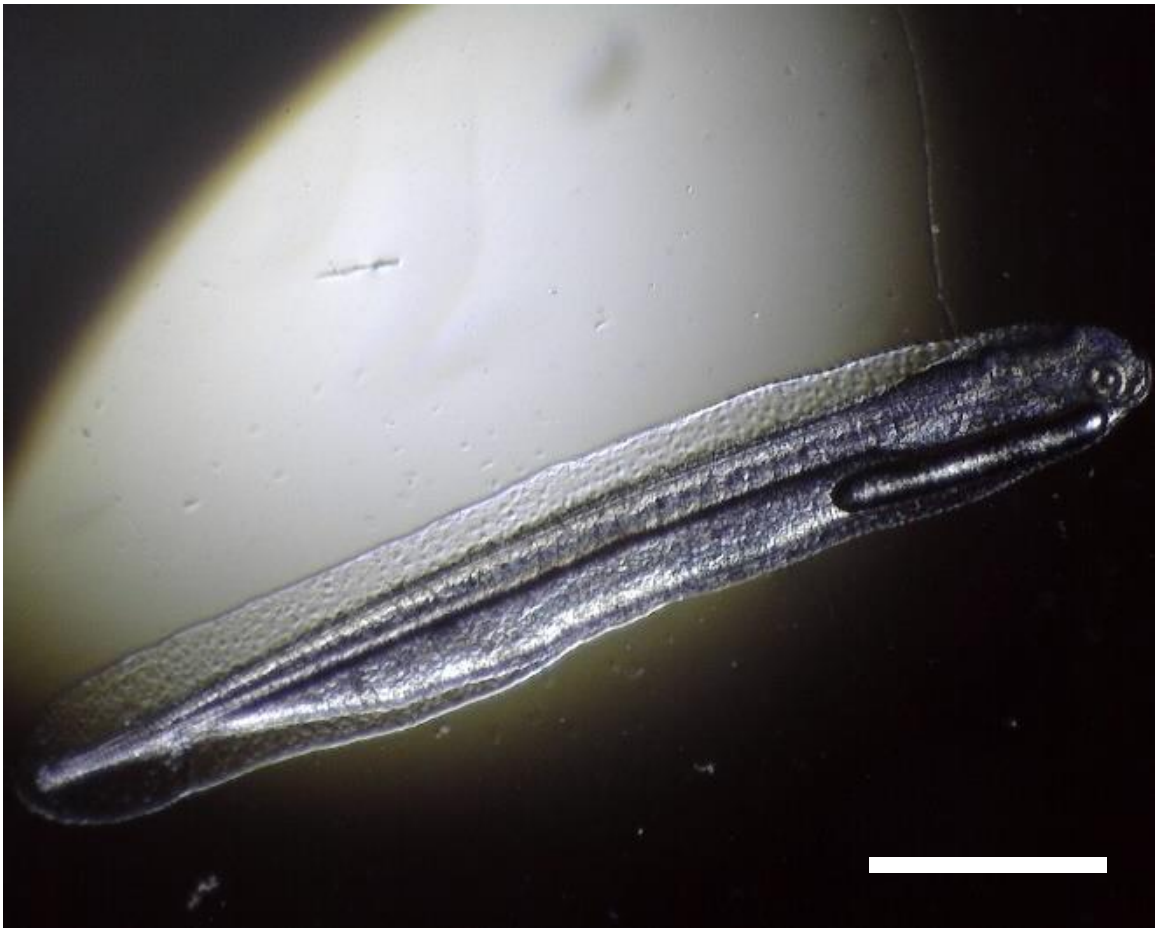

Figure 36. Larva imaged at CEI from Tank 1 at 10 hph. Image taken using darkfield lighting. Scale bar = 1 mm.

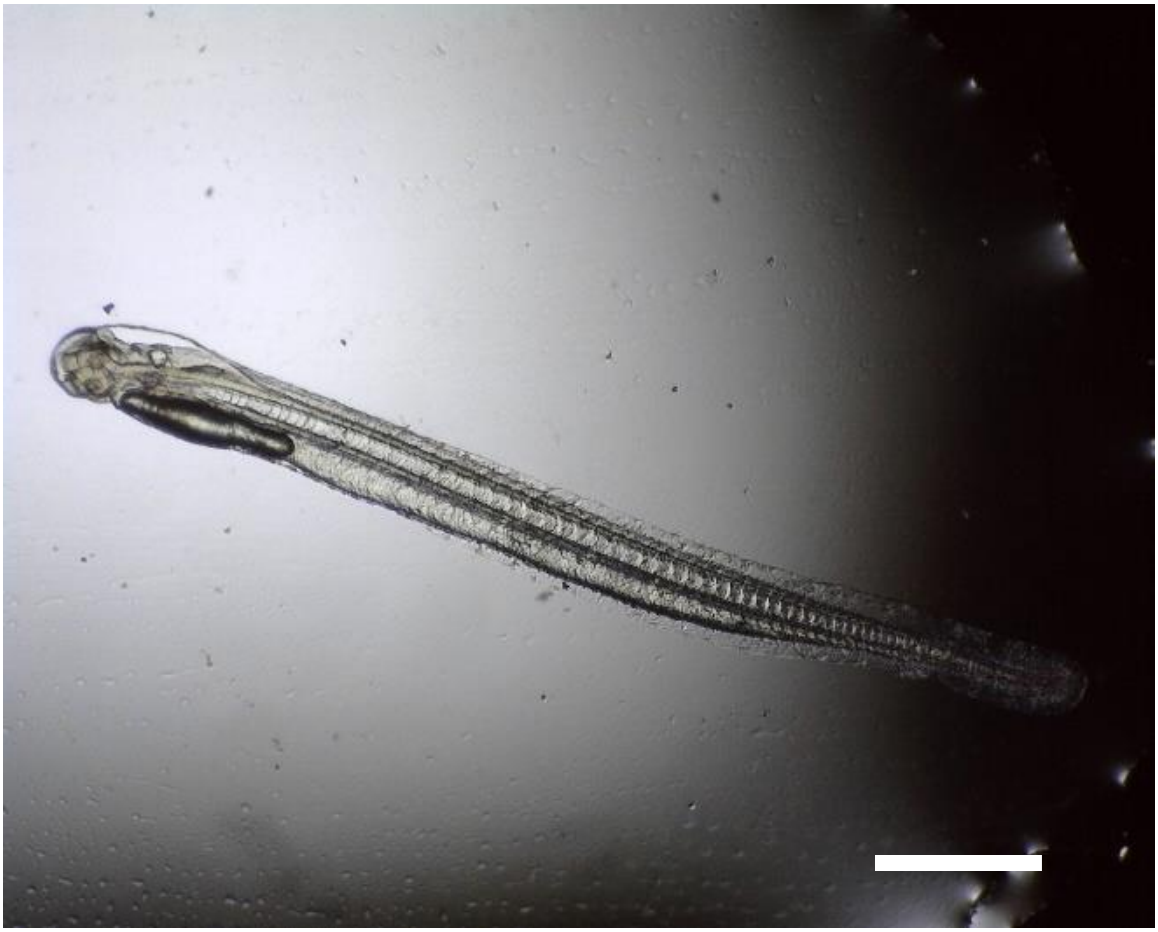

Figure 37. Larva imaged at CEI from Tank 2 at 30 hph. Image taken using darkfield lighting. Scale bar = 1 mm.

Figure 38. Larva imaged at CEI from Tank 2 at 42 hph. Image taken using darkfield lighting. No larvae in Tank 2 survived past 42 hph. Scale bar = 1 mm.

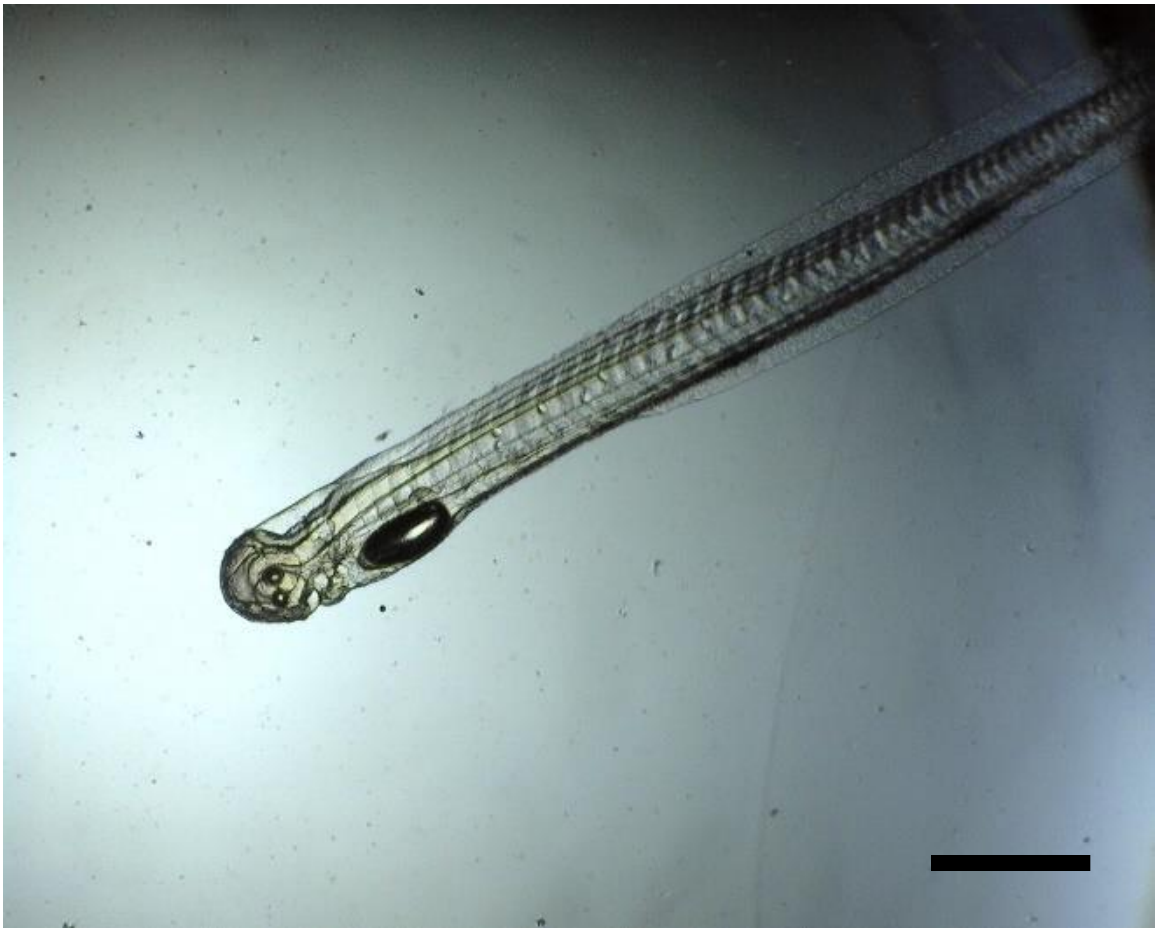

Figure 39. Larva imaged at CEI from Tank 1 at 52 hph. Image taken using darkfield lighting. No larvae in Tank 1 survived past 56 hph. Scale bar = 1 mm.

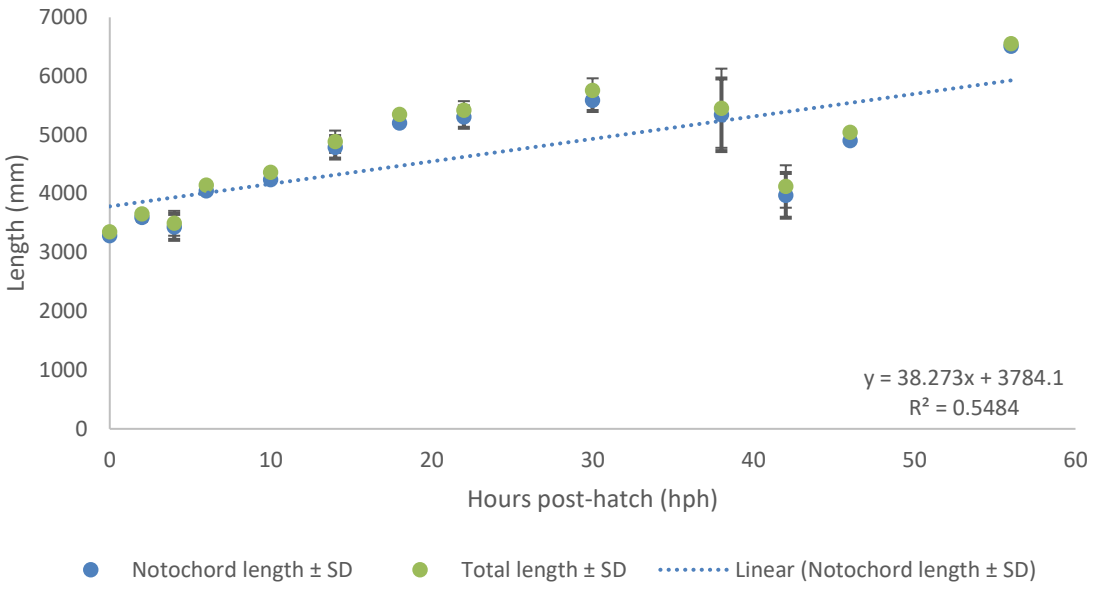

Figure 40. Mean lengths taken of fixed larvae from Tank 1. Notochord length has bold error bars. No larvae survived after 56 hph. N=1 for 6, 10, 22, 46 and 56 hph. Measurements could not be taken at 26, 34, and 52 hph. Linear regression is for notochord length and P<0.05, df(11).

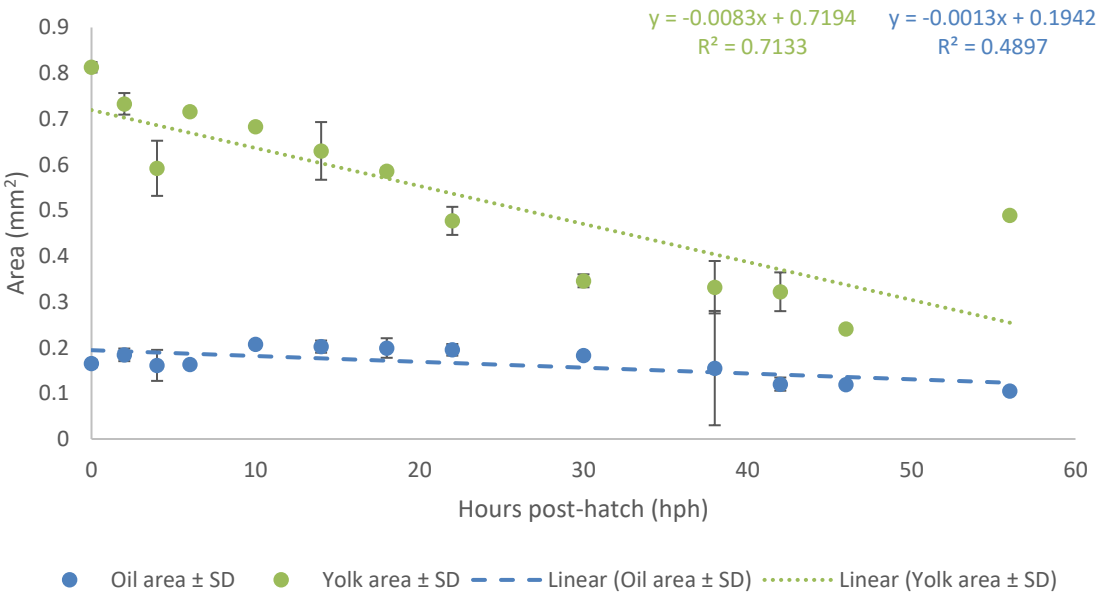

Figure 41. Mean oil and yolk areas taken of fixed larvae from Tank 1. No larvae survived after 56 hph. N=1 for 6, 10, 22, 46 and 56 hph. Measurements could not be taken at 26, 34, and 52 hph. P<0.01 for both linear regressions, df(11).

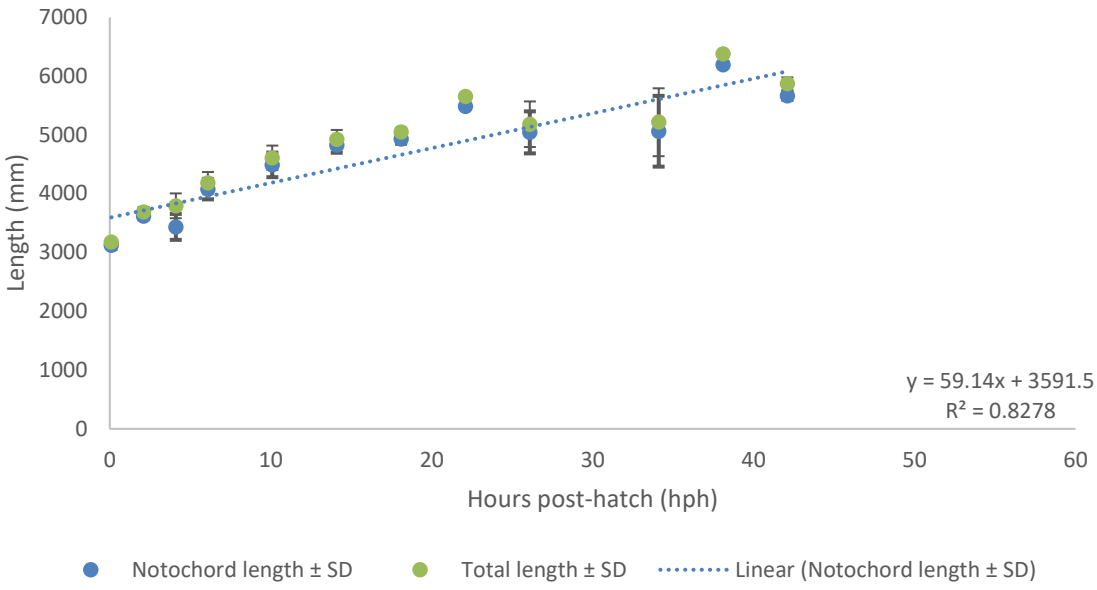

Figure 42. Mean lengths taken of fixed larvae from Tank 2. Notochord length has bold error bars. No larvae survived after 42 hph N=1 at 22 and 34 hph. Measurements could not be taken at 30 hph. Linear regression is for notochord length and P<0.001, df(9).

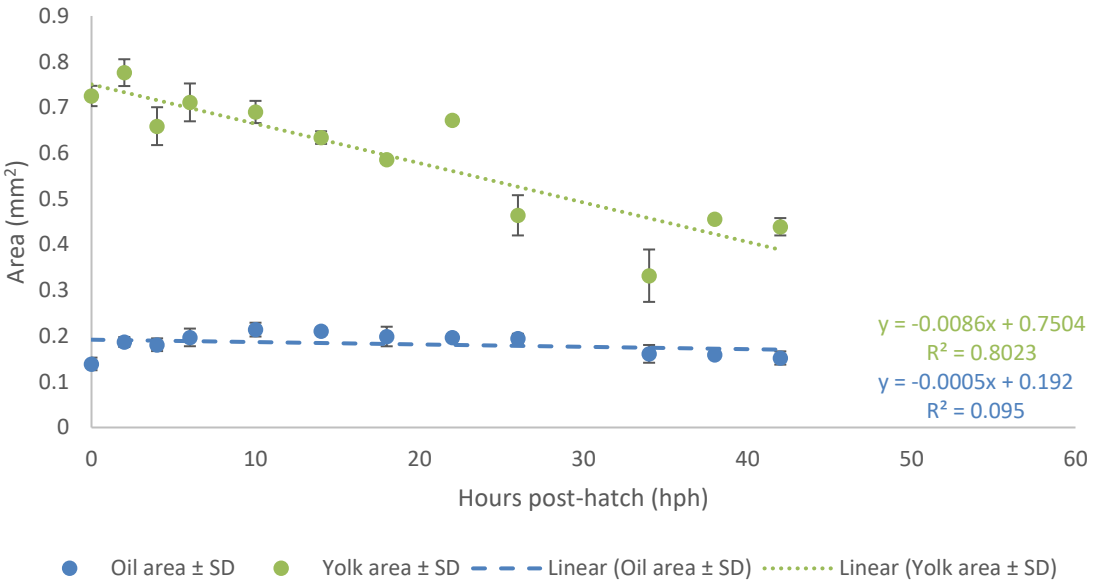

Figure 43. Mean oil and yolk areas taken in of fixed larvae from Tank 2. No larvae survived after 42 hph. N=1 at 22 and 34 hph. Measurements could not be taken at 30 hph. P<0.01 for both linear regressions, df(9).

Figure 44. Fixed larvae collected from Tank 2 at 0 hph. Scale bar = 0.5 mm.

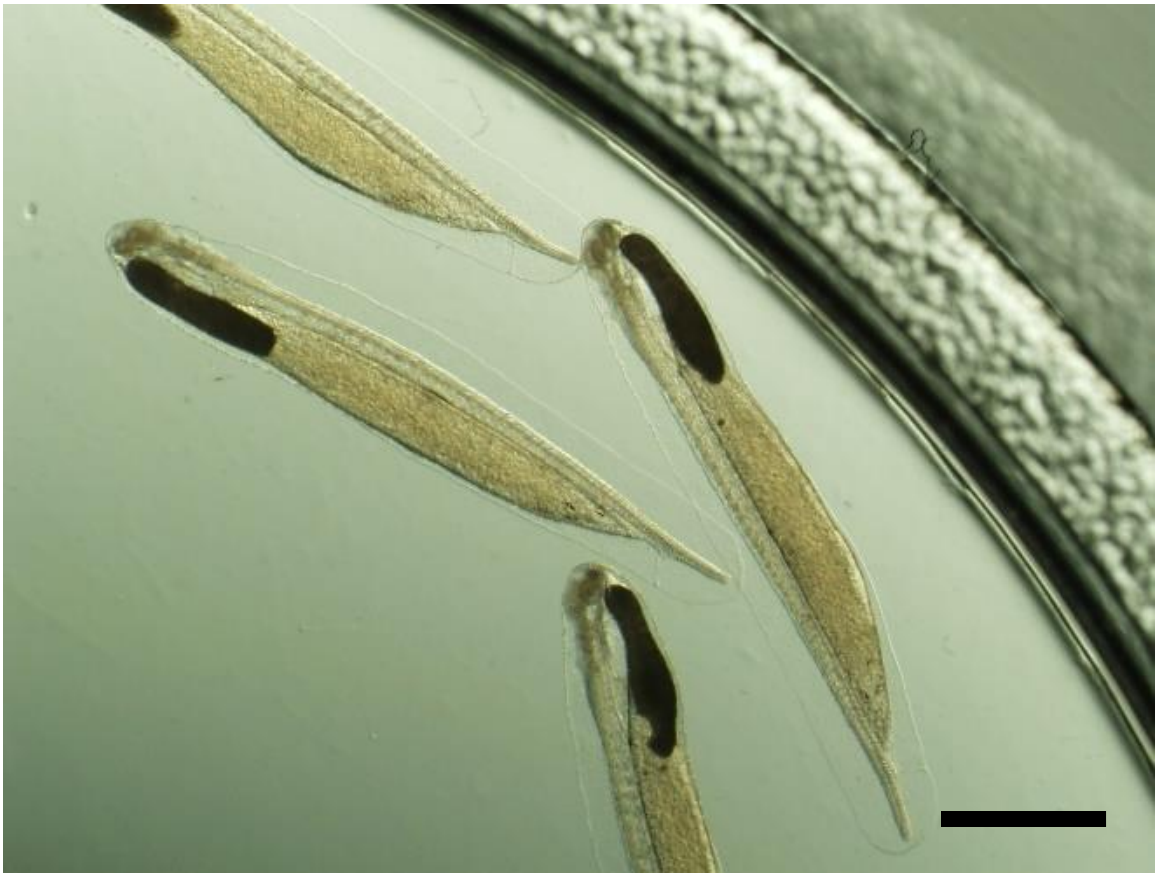

Figure 45. Fixed larvae collected from Tank 2 at 6 hph. Scale bar = 1 mm.

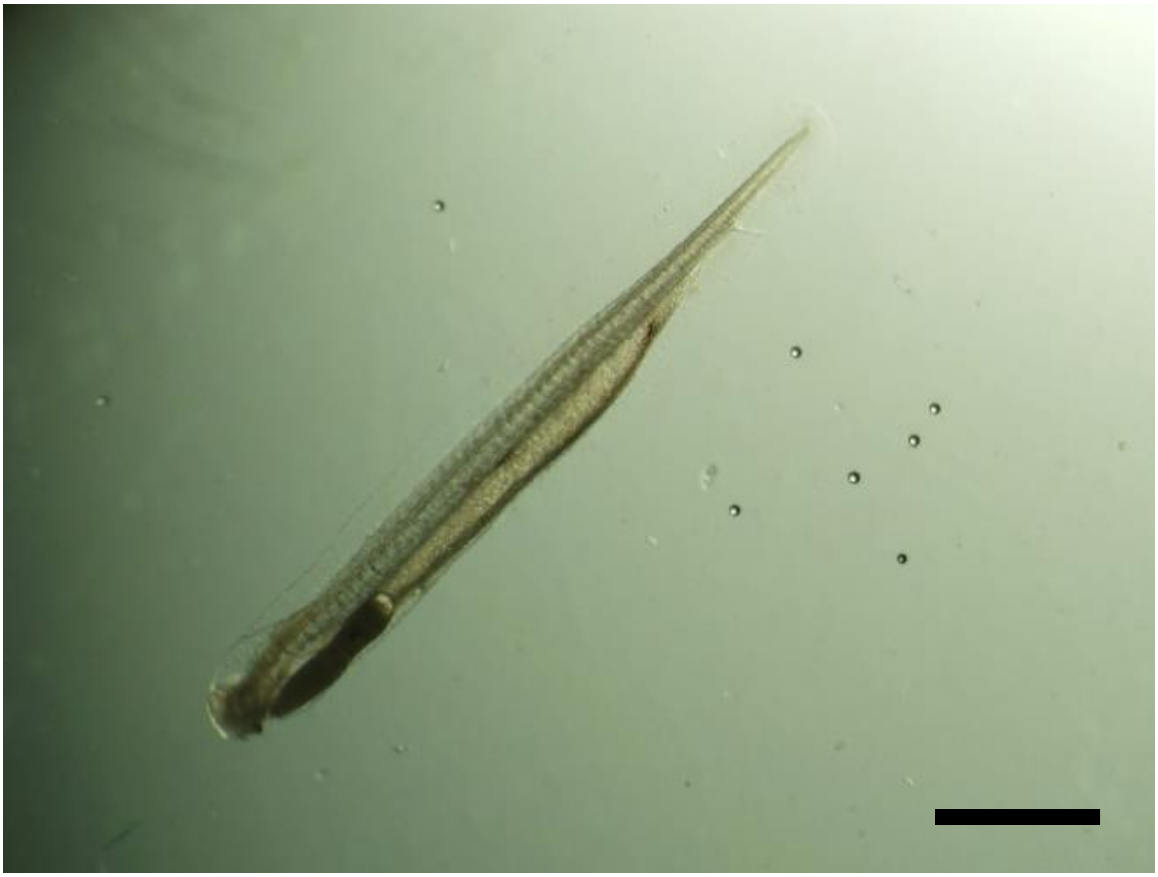

Figure 46. Fixed larva collected from Tank 1 at 22 hph. Scale bar = 1 mm.

Figure 47. Fixed larva collected from Tank 2 at 42 hph. No larvae in Tank 2 survived past 42 hph. Scale bar = 1 mm.

Figure 48. Fixed larva collected from Tank 1 at 56 hph. No larvae in Tank 1 survived past 56 hph. Scale bar = 1 mm.

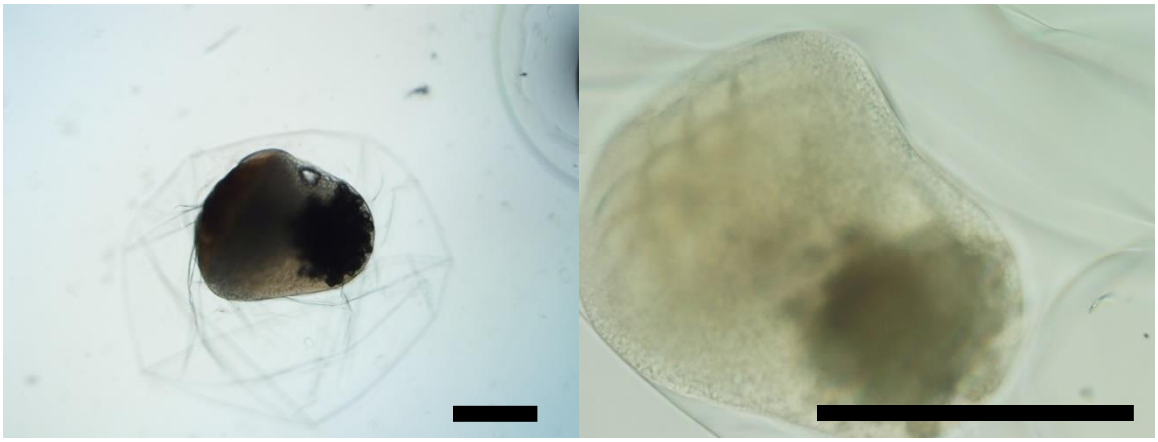

Figure 49. Fixed egg collected at 2 hpf, before and after clearing with 100% glycerol. Scale bars = 0.5 mm.

Figure 50. Unidentified polychaete collected with larvae in Tank 2 at 34 hph. Scale bar = 1 mm.

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