PUBLIC AQUARIUMS AS A POTENTIAL SOURCE OF MARINE FISH FOR EXHIBITS AND CONSERVATION

A Thesis

by

JESSIE L. CASTANIER

BS, Texas A&M University - Corpus Christi, 2014

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

The marine aquarium trade relies on wild fisheries for 98% of ornamental reef species for display. Overexploitation and harmful collection techniques threaten native fish populations and reef ecosystems. Public aquaria can contribute an untapped source of fishes by rearing eggs and larvae from volitional spawning events.

The aim of this study was to demonstrate that pelagic eggs and larvae collected from the mixed species Living Coral Reef (LCR) exhibit at the Texas State Aquarium (TSA) can be distinguished to species level based on unique morphological characteristics and barcoding. A floating collector was placed into the exhibit once per week for nine weeks to collect eggs and larvae from volitional spawning events. Fin clips were obtained from adult fishes in the exhibit for genetic reference and preserved in 20% salt-saturated dimethylsulfoxide (DMSO) until processing. Eggs and larvae were identified molecularly using DNA barcoding at the CO1 sequence region.

Eggs of smallmouth (*Brachygenys chyrsargyreum*) and cottonwick (*Haemulon melanurum*) grunts were genetically identified and showed significantly different ($p > 0.0001$) mean egg diameters (± s.d.) of 860 ± 45 µm and 972 ± 30 µm, respectively. Bicolor damselfish (*Stegastes partitus*) larvae, 1 day post hatch, were recognized by characteristic brain, stomach, and ventral pigmentation.

This work provides a verification of techniques for collecting eggs and larvae in public aquariums. Identification of eggs and larvae in mixed species exhibits may allow for more efficient sorting and subsequent larval culture. Further research can increase conservation efforts in public aquaria by strengthening the capacity to promote sustainable sources of marine ornamentals through exhibits and outreach.

DEDICATION

This manuscript is dedicated to everyone that has guided me throughout life's journey. My family has provided endless encouragement and support. My dearest sister, Jaidyn Turner, has been my greatest inspiration. My mother, Bernadette, and father, James Turner, have planted their strength and work ethic deep within me. My aunt, Janet Collis, has taught me persistence by demonstrating her unlimited resilience every day. My brothers Michael, John, and Chris Turner have been notable role models in my life. I would like to thank Kelsey and Michael Turner for encouraging my pursuits in science and supporting my eccentricity. My dedication goes to Megan, and John Turner for their unwavering acceptance, and their extraordinarily compassionate sons, Will, Kayden, and Milo. This work is also dedicated to my cherished friends and family, Lauren Meza, Marie, Mark, and Caleb Gonzalez, Sandra, Dudley, and Alex Austin, as well as Kevin and Eulalia Juniper de Santiago.

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This project would not be possible without the unrelenting encouragement of my manager, Kara Hahn. I would also like to extend my gratitude to Sean Hardegree for his guidance with project development and life support logistics. A special thanks goes to my sister, Jaidyn Turner, and Andrew Johnson for assisting with sampling and data collection. A huge thank you goes to my friends, Aline Trejo-Chavez, and Patricia Malamalama Cockett, MS, for their invaluable expertise in technical writing and coding. Finally, I would like to acknowledge Alex Austin, MS, Alexa Cano, and Nicole Gaertner, MS, for assistance with editing, formatting, and conceptualization.

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CHAPTER I

INTRODUCTION

Wild-caught reef fishes account for 98% of all marine ornamentals in the aquarium industry (Smith et al. 2008; Donnelly 2010; Rhyne et al. 2012; Holcombe et al. 2022). Destructive methods of capture often used for marine ornamentals can cause significant damage to ecologically important coral reef habitats (Gopakumar 2004; Rubec and Cruz 2005; Olivotto et al. 2017). Sodium cyanide is a toxic chemical dispersed into the water to stun fishes, making them easier to collect. The use of sodium cyanide in fisheries, prohibited by 72% of exporting countries and territories (Dee et al. 2014), is difficult to detect as it is rapidly metabolized by fishes (Gopakumar 2004; Rhyne et al. 2009). Overexploitation of marine fishes from bycatch (Feitosa et al. 2008), rejection, and mortalities during capture (Gopakumar 2004; Rubec and Cruz 2005) and transport (Rubec and Cruz 2005; Sampiao et al. 2016) can also contribute to declines in wild fish abundance (Olivotto et al. 2017; Dee et al. 2019).

Compounding these problems, marine ornamental fishes for public and private aquaria are primarily sourced through ambiguous supply chains (Wood 2001; Rubec and Cruz 2005; Teletchea et al. 2006; Smith et al. 2008; Donnelly 2010; Murray et al. 2012; Rhyne et al. 2012; Allen et al. 2017; Rhyne et al. 2017; King 2019; Pinnegar and Murray 2022), making it difficult to create change by preferentially using “sustainable” suppliers. Furthermore, suppliers are prone to misidentifying species and are not required to provide the source location of where fish are captured (Rubec and Cruz 2005; Smith et al. 2008; Murray et al. 2012; Allen et al. 2017; Pinnegar and Murray 2022). Shipping labels require minimal documentation with only 4% of shipments identified to the family, genus, or species level (Murray et al. 2012; Rhyne et al. 2012; Allen et al. 2017). Incongruent import and export data misrepresent not only the species traded,

but the number of individuals of each species, as well (Rubec and Cruz 2005; Smith et al. 2008; Murray et al. 2012; Rhyne et al. 2012; Allen et al. 2017; King 2019).

Although the International Union for Conservation of Nature (IUCN) has not evaluated the status for most of the top marine aquarium fish on the IUCN Red List of Threatened Species, (Dee et al. 2014), reef ecosystems are listed as critically endangered. The conservation and management practices utilized for food fisheries can be applied to reef habitats (Dee et al. 2014). Sustainability assessments of marine ecosystems have examined the effects of ornamental fisheries on individual species, as well as the impacts on local diversity (Bruckner 2005; Feitosa et al. 2008; McCauley et al. 2008; Reynolds et al. 2008; Allen et al. 2017). Feitosa et al. (2015) examined conservation techniques that minimize the effects of harvest and bycatch on populations of French angelfish (*Pomacanthus paru*), one of the five most exported ornamental species from Brazil. Fisheries management strategies for French angelfish include controlling the number of individuals harvested, monitoring catch rates, and designating trap-free areas in spawning grounds (Feitosa et al. 2015). Continuous assessments of the vulnerability of reef fish populations, in a changing climate, can aid in designating marine protected areas known to be utilized during spawning (Amaral and Jablonski 2005; McCauley et al. 2008; Reynolds et al. 2008; Donnelly 2010). However, local fisheries managers are limited by effective communication and enforcement of regulations in a market with high demand for wild caught ornamentals (Bruckner 2005; Donnelly 2010; Murray and Watson 2014; Teletchea 2016; Rhyne et al. 2017a; Rhyne et al. 2017b).

Conversely, aquaculture of desirable species can reduce pressures on local ornamental fisheries, decreasing the number of wild-caught individuals (Ziemann 2001; Monteiro-Neto et al. 2003; Dominguez and Botella 2014; Murray and Watson 2014; Olivtotto et al. 2017; Buckley et

al. 2018; Dee et al. 2019; King 2019). Culture of ornamentals decreases the exploitation rate on threatened fish populations by reducing the number of fish removed from the wild as well as mortalities from capture, rejection (Militz et al. 2016), transport (Sampiao et al. 2016), acclimation, and disease (Tlusty 2002). Rearing fish from eggs or larvae also minimizes the shipping and treatment costs associated with wild-caught individuals (Tlusty 2002).

Although recent progress has been made in culturing tropical marine fish, only 4% of marine aquarium species have been reared in captivity (Holcombe et al. 2022). Successes in larval rearing can be attributed to the application of techniques, such as live feed schedules, used for culturing foodfish (Ostrowski and Laidley 2001). Commonly cultured marine ornamentals, such as clownfish and damselfish, now have established commercial-scale larval rearing protocols (Gopakumar et al. 2009; Rajasekar 2009). Various factors, such as ease of capture or culture, conservation status and marketability, determine the feasibility of candidates for larval culture research and design (Dee et al. 2014; Dee et al. 2019; Holcombe et al. 2022). Callan et al. (2018) demonstrated commercial-scale production protocols through optimized feed techniques with one of the most popular marine aquarium species, the yellow tang (*Zebrasoma flavescens*).

Partnerships with academia, aquaculture farms, and public aquariums have led to most of the recent advances in ornamental fish aquaculture (Olivotto et al. 2011; Pouli et al. 2015; St. Leger and Violetta 2017). Volitional or natural spawning that occurs in aquariums can be used to develop improvements in aquaculture research and system design by capturing and utilizing pelagic eggs and larvae that would have otherwise been lost to predation and filtration (Cassiano et al. 2015; Calado 2017; Chen et al. 2019). Public aquariums have provided an invaluable medium for educating the public about the importance of marine wildlife and ecosystem conservation (Hall and Warmots 2008; Holt et al. 2017). Furthermore, there is potential for

aquariums to develop outreach programs that inform community members about larval fish development and the important role that fishes in ornamental trade play in maintaining coral reef ecosystem health.

The Association of Zoos and Aquariums (AZA), a global leader in promoting species conservation and animal welfare, offers an accreditation and partnership among facilities, stakeholders, and Taxon Advisory Groups (TAGs) working towards conservation management plans and self-sustaining captive populations (Hall and Warmolts 2008). Aquariums can maximize conservation impact by participating in Species Survival Plan (SSP) programs, implementing Institutional Collection Plans (ICPs), providing educational exhibits, and emphasizing the importance of research in science (Hall and Warmolts 2008). Rising Tide Conservation (RTC), created by SeaWorld Parks and Entertainment in 2009, is a collaborative program to promote economic aquaculture for marine ornamental fish conservation (Cassiano et al. 2015). Partnerships in RTC among researchers, industry workers, and AZA accredited facilities have led to advancements in larval rearing (Cassiano et al. 2015). The University of Florida's Tropical Aquaculture Laboratory (UFTAL) in Ruskin, FL successfully developed aquaculture protocols for French grunt (*Haemulon flavolineatum*), among other species, using eggs collected from Epcot's The Seas exhibit (Barden et al. 2014; Hauville et al. 2017). Protocols for raising porcupine puffer (*Cyclichthys orbicularis*) were established at the Shimonoseki Marine Science Museum Aquarium in Shimonoseki, Japan (Doi et al. 2015).

Identifying eggs and larvae collected from mixed species exhibits in aquariums can be challenging with overlapping spawns and minimal information on egg and larval morphology (Cassiano et al. 2015). Biodiversity assessments, utilized in the field with wild populations and in hatcheries for stock enhancement, can be applied to identify eggs and larvae in aquariums

(Murray and Watson 2014; Cassiano et al. 2015). DNA barcoding paired with morphological studies has been used to describe early life history stages of many species of fish and provide tools that could be used in aquarium settings (Richards 2005; Aranishi 2006; Hyde et al. 2006; Kawakami et al. 2010; Sme 2012; Cassiano et al. 2015).

The Aquarium Sustainability Program is a formal research partnership created with Roger Williams University (RWU) in Bristol, RI and the New England Aquarium (NEA) in Boston, MA. Initiatives from the program include the Larval Culture Project, focused on reducing the need for public aquariums to collect animals from the wild. Researchers at RWU created an identification protocol for aquariums using eggs and larvae collected from the Giant Ocean Tank (GOT) at the NEA and the Pacific Barrier Reef exhibit at the Georgia Aquarium (Sme 2012). The study resulted in a catalog of fish eggs and larvae for 20 species including blue chromis (*Azurina cyanea*), brown chromis (*Azurina multilineata*), white grunt (*Haemulon plumeria*), bluestriped grunt (*Haemulon sciurus*), redband parrotfish (*Sparisoma aurofrenatum*), stoplight parrotfish (*Sparisoma viride*), longfin damselfish (*Stegastes diencaeus*), and bicolor damselfish (*Stegastes partitus*), in addition to providing the first documentation of spawning in captivity among ocean surgeon (*Acanthurus bahianus*), foureye butterflyfish (*Chaetodon capistratus*), white grunt, and others (Sme 2012). Research at the UFTAL, with the University of Florida's Wildlife and Aquatic Animal Veterinary Disease Laboratory (WAVDL), identified eggs and larvae collected from the Birch Aquarium (BA) in San Diego, CA, Columbus Zoo and Aquarium (CZA) in Powell, OH, Discovery Cove (DC) in Orlando, FL, and John G. Shedd Aquarium (SA) in Chigaco, IL using DNA barcoding to document morphology (Cassiano et al. 2015). The study resulted in larval rearing protocols for bannerfish (*Heniochus sp.*), Pacific blue

tang (*Paracanthurus hepatus*), and semicircle angelfish (*Pomacanthus semicirculatus*) (Cassiano et al. 2015; DiMaggio et al. 2017).

Most of the information on life history and morphology of fishes originated from field studies (Hildebrand and Cable 1930; Kendall et al. 1984; Matarese and Sandknop 1984; Powels and Markle 1984; Sandknop et al. 1984; Sumida et al. 1984; Hyde et al. 2006). The morphological variables used to describe eggs and larvae collected during these and subsequent studies included egg diameter, and larval pigmentation and total length (TL) (Kelly 1995; Alshuth et al. 1998; Richards 2005; Leu et al. 2009; Cassiano et al. 2015; DiMaggio et al. 2017). Other characteristics examined included the number of oil globules in the egg, diameter of the oil globules, oil globule coloration, and pigmentation of the embryo (Matarese and Sandknop 1984; Moser 1996; Alshuth et al. 1998; Richards 2005; Leu et al. 2009; Kawakami et al. 2010; Callan et al. 2012; Sme 2012). Olivotto et al. (2006) also examined these features in documenting the spawning, development, and larval rearing of lemonpeel angelfish (*Centropyge flavissimu*s). Similarly, Callan et al. (2014) measured egg diameter and oil globule diameter of flame angelfish (*Centropyge loriculus*), examining how hatch rate, length at hatch, length at day three, and survival to day three is affected by maternal nutrition. Callan et al (2014) found egg production and egg quality increased in flame angelfish eggs when broodstock were fed raw and highly unsaturated fatty acid (HUFA)-rich diets in comparison to formulated feeds, while morphological egg measurements remained constant. DiMaggio et al. (2017) measured embryo diameter, as well as standard length (SL) and TL of larvae, from hatch to 54 days post hatch (dph) to establish optimal diets for culture of Pacific blue tang.

The identities of eggs and larvae collected from the field, or from mixed species exhibits, can be confirmed using genetic barcoding, i.e., sequencing the mitochondrial cytochrome

oxidase 1 (CO1) gene region (Ward et al. 2005; Aranishi 2006; Ivanova et al. 2007; Rhyne et al. 2009; Steinke et al. 2009; Kawakami et al. 2010; De Oliveira Ribeiro et al. 2012; Sme 2012; Ko et al. 2013; Cassiano et al. 2015; Rabaoui et al. 2019). Mitochondrial DNA is extracted from fish tissue and 655 base pair (bp) of CO1 region is amplified using polymerase chain reaction (PCR) and universal primers that work broadly across fishes (Ward et al. 2005; Aranishi 2006; Ivanova et al. 2007; Rhyne et al. 2009; Steinke et al. 2009; Kawakami et al. 2010; De Oliveira Ribeiro et al. 2012; Sme 2012; Ko et al. 2013; Cassiano et al. 2015) and Sanger sequenced. Results from sequencing allowed comparison of DNA from samples to those available in the GenBank database or on Barcode of Life. Results obtained using the National Center for Biotechnology Information Nucleotide Basic Local Alignment Search Tool (NCBI BLASTN) include percent matches and e values. Species identity can be confirmed by comparing the nucleotide sequences of unknown eggs and larvae to sequences of known species in GenBank and choosing those with optimal e values.

The purpose of the present study was to determine if eggs and larvae collected from the Living Coral Reef (LCR) exhibit at the Texas State Aquarium (TSA) in Corpus Christi, TX could be accurately identified to the species level based on morphological characteristics. The identity of each species was confirmed using genetic barcoding. Objectives of this study were to 1) collect samples of fish eggs and larvae from different volitional spawning events in the LCR exhibit at TSA, 2) measure and describe morphological parameters of eggs and larvae, 3) determine the species of eggs and larvae by DNA barcoding, 4) determine if measured aspects of egg morphology could be used to discriminate fish species, and 5) construct a fish species key with identifying morphological features.

CHAPTER II

METHODS

Pelagic eggs and larvae were collected from the Caribbean themed Living Coral Reef (LCR) exhibit at the Texas State Aquarium (TSA), Corpus Christi, Texas. Eggs and larvae were morphologically examined using a stereomicroscope, photographed, and measured. Although several egg characteristics, including egg diameter, number of oil globules, oil globule diameter, oil globule color, and pigmentation of the embryo were evaluated, egg diameter was the only consistent and reliable feature of eggs genetically identified to species. Larvae were presumptively identified by total length (TL) and pigmentation. DNA was extracted (Chelex; Bio-Rad) from the samples and the CO1 region was Sanger sequenced. Nucleotide sequences of egg and larval samples were compared to those from fin clips taken from adults in the LCR exhibit and GenBank sequences to identify to species level.

Sample Collection

The organisms in this study were collected from the LCR exhibit (Fig. 1), home to 20 species of teleost (Table 1). Courtship behaviors are often observed at dusk and dawn among several species including French angelfish (*Pomacanthus paru*), blue tang (*Acanthurus coeruleus*), squirrelfish (*Holocentrus adscensionis*), and volitional spawning occurs regularly in the exhibit (pers. observ.). The LCR system contains 68,000 gallons of marine water and shares a life support system with the Blue Hole exhibit (Fig. A.1). Pelagic eggs and larvae, along with other floating debris and organisms in the water, are removed by skimmers on the top of the exhibit. The skimmers feed into three pumps and four sand filters. The system water flows through two foam fractionators and a contact chamber for ozonation. Ozonated water is degassed in a degas chamber before returning to the exhibit (Fig. A.1).

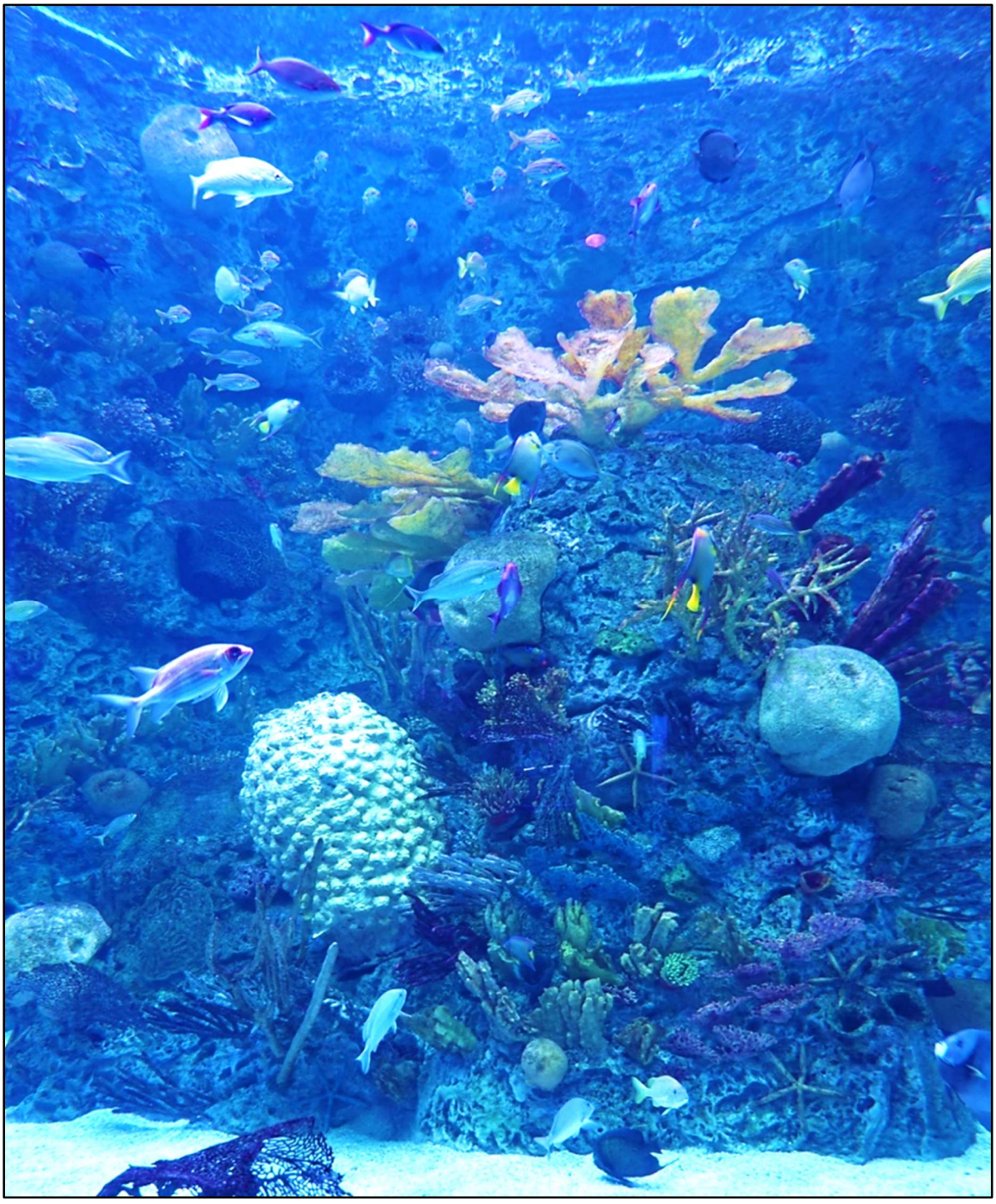

Figure 1: Living Coral Reef exhibit at the Texas State Aquarium.

Table 1: Species of the Living Coral Reef exhibit at the Texas State Aquarium.

Scientific Name	Common Name
Acanthurus coeruleus	Blue tang
Acanthurus bahianus	Ocean surgeonfish
Aluterus scriptus	Scrawled filefish
Aluterus shoepfii	Orange filefish
Bodianus pulchellus	Spotfin hogfish
Bodianus rufus	Spanish hogfish
Azurina cyanea	Blue chromis
Clepticus parrae	Creole wrasse
Brachygenys chyrsargyreum	Small mouth grunt
Haemulon flavolineatum	French grunt
Haemulon melanurum	Cottonwick grunt
Holacanthus bermudensis	Blue angel
Holacanthus ciliaris	Queen angel
Holocentrus adscensionis	Squirrelfish
Mulloidichthys martinicus	Yellow goatfish
Paranthias furcifer	Creolefish
Pomacanthus arcuatus	Gray angel
Pomacanthus paru	French angel
Stegastes adustus	Dusky damselfish
Stegastes partitus	Bicolor damselfish

A floating egg and larvae collector (Ohs et al. 2019) was placed into the LCR exhibit in the evening (~1800 hours; Fig. 2). Pelagic eggs and larvae, normally removed through filtration within a few hours of spawning or hatch, were pulled into the container through an outer PVC pipe by airlift suction (Fig. 2). The top skimmers of the exhibit were discontinued in the evening until after sample collection the following morning (~0800 hours). Eggs and larvae were scooped from the collector with 1000 mL glass beakers.

Individual eggs and larvae were pipetted into a square watch glass (1.6 x 1.6 x 0.6 in) and then transferred to 1.5 mL sample tubes with 20% salt-saturated dimethyl sulfoxide (DMSO) buffer for preservation (Dawson et al. 1998; Gordeeva et al. 2019). The eggs were approximately 10 to 13-hours post-spawn, and larvae were estimated to be 1 day post hatch (dph) when preserved. A minimum of 30 individual eggs and 30 individual larvae were collected 1-2 times

per week for nine weeks on the following dates: May 23, 30, June 6, 13, 20, 27, July 4, 11, and 18 of 2020. A total of 258 egg and 129 larval samples were morphologically analyzed. Genetic information was collected for 19 larvae and an additional subsample of 31 eggs from the original collection period. Collection methodology was tested and revised during preliminary sampling in November of 2019, resulting in modified protocol for minimizing morphological damage when removing eggs and larvae from the collector.

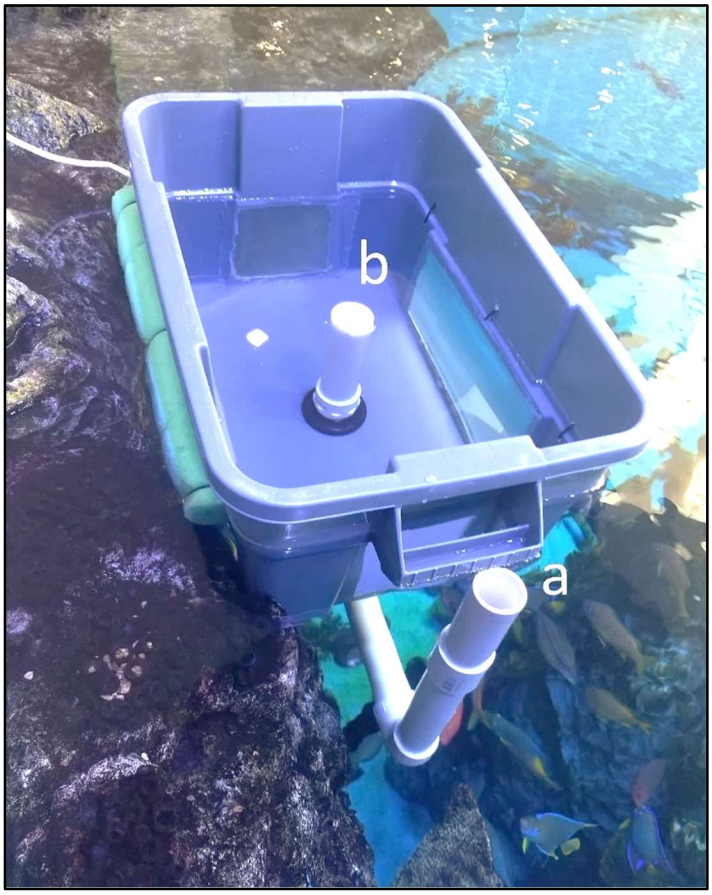

Figure 2: Egg and larva collection basket with a) influent and b) effluent (airlift not shown) from the Living Coral Reef exhibit.

Fin clip tissue was collected, based on which species aquarists were able to catch, from 11 of the 20 fish species in the LCR exhibit. Fishes were taken from the exhibit a few days before fin clip sampling and placed in holding tanks maintained at 30 ppt, 21°C, and pH of 8. Veterinary administered anesthesia (80 ppm Tricaine methanesulfonate, or MS-222, buffered

with sodium bicarbonate) was used to reduce stress on the fish during sampling. Oxygen was supplemented and dissolved oxygen was maintained at 100-110% throughout the procedure. Individual fish were placed in the anesthesia solution for approximately five minutes or until equilibrium was lost.

Each fish was cataloged, photographed, and fin-clipped within five minutes after anesthetization. Fin clip samples (approximately 5 mm in length) were taken with sterilized surgical scissors from a small section of the posterior region of the pelvic fin (Fig. 3) to minimize the impact on mobility and aesthetics of the fish. Each fish was awakened from anesthesia by being placed into a container of system water and was watched until regaining normal functions (usually within 5 minutes). The fish were returned to the original tanks soon after recovery. Fin clip tissues were placed in 2 mL vials with 1.5 mL of salt saturated DMSO buffer and stored at room temperature (Gordeeva et al. 2019).

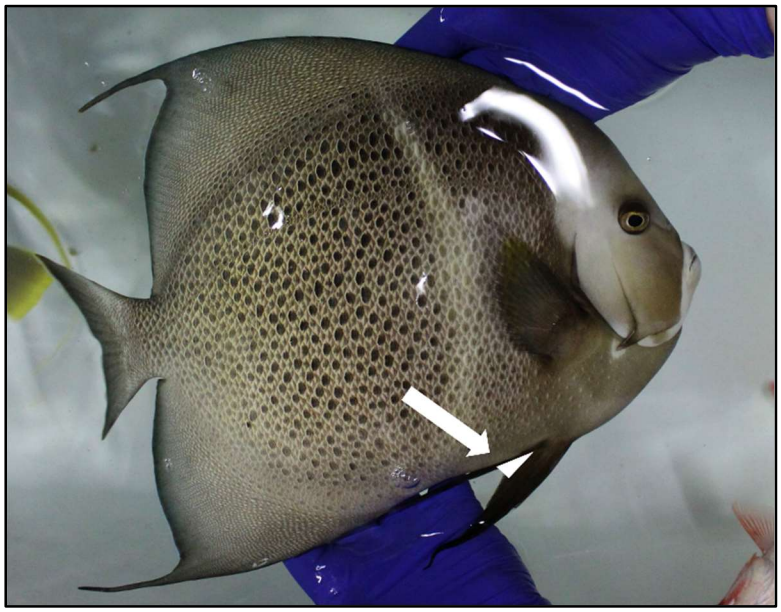

Figure 3: Anesthetized adult grey angelfish (*P. arcuatus*) and fin clip tissue sampling area indicated by white triangle and arrow.

Morphology

Egg and larvae were pipetted into a high-rimmed glass culture dish watch glass (1.5 in, 6 mL) containing enough DMSO to cover the depth of the eggs and larvae. Individuals were photographed using a ZIESS SteREO Discovery.V8 stereomicroscope and AxioImager with Zeiss Axiocam 506 mono, and a Stemi305 stereomicroscope with Axiocam ERC5s (Carl Zeiss Microscopy, LLC, NY, USA). Measurements were made from photographs of eggs and larvae using ZEN software (Carl Zeiss Microscopy, LLC, NY, USA). Data recorded for each egg included egg diameter, number of oil globules inside of the egg, diameter of oil globules, and oil globule coloration.

A morphologically diverse subsample of eggs (n = 31) from the original sampling period was chosen for genetic analysis using size categories and number of oil globules. Size groupings for egg categories (<880, 880-980, and >980 μm) were based on the frequency plot of egg diameter (Fig. 4). Larvae were photographed, laid down flat, and oriented on the left side. Total length (TL) and pigmentation was recorded for each larva (Alshuth et al. 1998; Richards 2005; Leu et al. 2009; Kawakami et al. 2010; Sme 2012; DiMaggio 2017). Whole eggs, larvae, and fin clip tissues were preserved in DMSO buffer and stored at 12°C during sample preparation, or 4°C for long term preservation. Larvae were identified using the illustrations and descriptions in "Early Stages of Atlantic Fishes" (Richards 2005).

Genetic Barcoding

DNA selected for sequencing included 11 adult fin clip samples preserved in DMSO buffer. A single egg and 11 larvae (refrigerated at 12°C in seawater) from preliminary sampling (November 4, 2019) were sequenced while establishing experimental protocol. A total of 258 eggs and 129 larvae from the nine-week sampling period were morphologically analyzed and

prepared for CO1 sequencing. However, extracted DNA was left at 12°C for several months during sample preparation and sequences were only obtained for a single egg and 8 larvae. A subsequent sample of eggs (n = 31) from the same collection period was then selected and successfully sequenced.

DNA was extracted according to a modified protocol that accommodates small-mass (µg) samples adapted from the Texas A&M University-Corpus Christi Marine Genomics Lab (Aranishi 2006; Kawakami et al. 2010). The initial incubation of 500 µL of Chelex resin (10%) was done at 60°C for one hour, and Chelex was added to the tubes while on a stir plate. All samples chosen for DNA extraction were briefly blotted with a clean Kimtech Wipe ™ to remove extra DMSO buffer. Egg and larval samples were processed whole while only a piece of fin clip tissue (approximately 1 mm in length) was used for each DNA extraction. Next, clean and macerated tissues were added to preheated Chelex along with 28 µL of Proteinase K (10 mg/mL). Each egg was macerated in the tube with sterilized forceps according to Kawakami et al. (2010). Finally, all samples were incubated at 60°C (1.5 hours), then at 95°C for 15 minutes to complete DNA extraction. The clear supernatant containing the DNA was transferred by pipette into a clean 0.5 mL PCR tube and stored at 4°C.

After successful genomic DNA (gDNA) extraction, a modified PCR protocol (GoTaq Flexi DNA Polymerase Kit; Promega) was used to amplify the target mitochondrial cytochrome oxidase (CO1) gene following Palumbi et al. (1996) and Ward et al. (2005). Extracted DNA (1 µL) was added to each PCR master mix (PCR MM) for a total reaction volume of 30 µL (in two 50 mL falcon tubes). Each set of PCR reactions included a negative control (1 µL of molecular grade water containing no DNA) and a positive control (1µL of gDNA of known quantity and size). PCR MM was prepared using the GoTaq Flexi DNA Polymerase Kit. Each reaction

contained, 5X green GoTaq Flexi reaction buffer (1 M), magnesium chloride (MgCl2; 1.5 M), 1% Tween (polyethylene glycol sorbitan monolaurate; 0.04 M), dNTPs (0.2 M), primers FishF1 (0.25 M), FishR1 (0.25 M), FishF2 (0.25 M) and FishR2 (0.25 M), GoTaq DNA polymerase (0.03 M) and water. After vortexing the PCR MM, GoTaq DNA polymerase and 1 µL of sample DNA was added separately to each PCR tube and mixed by pipette. A Fisher Thermocylcer™ was used to execute the PCR reaction with an initial high denaturation at 95°C (2 min), followed by 35 cycles of denaturation at 95°C (1 min), annealing at 50°C (1 min), extension 72°C (1.5 min); then a final extension at 72°C (10 min) followed by a holding temperature of 4°C.

Amplified mitochondrial DNA (mtDNA) from the target CO1 region, approximately 650 base pairs (bp), was assessed, and quantified for samples. A total of 3 µL PCR product was visualized after electrophoresis using an agarose gel (3%; 90 v; 200 AMP; 60 min). DNA ladders (1.5 µL of 100 bp; Omega Bio-Tek, Inc., GA, USA) were used to estimate size of amplicons. The gel was viewed using the UVP GelDoc-It2 Imager (Analytik Jena US LLC, CA, USA) and photographed with VisionWorksLS Image Acquisition and Analysis Software (Analytik Jena US LLC, CA, USA) with UV transillumination. Successful amplification of the targeted CO1 gene region was indicated by the presence of bands between 650 and 750 bp.

Amplified mtDNA was purified using AMPure (Mag-Bind Total Pure NGS, Omega Bio-Tek, Inc., GA, USA). The AMPure master mix (AMP MM) was prepared according to manufactures instructions. DNA purification was done with an 80% ethanol (EtOH) wash (100 µL per sample reaction) and DNA was eluted into 20 µL of molecular grade water. The final concentration (ng) for cleaned mtDNA samples was quantified using the Qubit dsDNA HS Assay Kit with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Samples with high

concentrations of DNA (i.e., >20 ng/μL) were diluted (1:1 DNA and molecular grade water) then re-quantified. Final concentration range for all samples was between 5 and 20 ng/μL.

Sequencing of CO1 was done by Retrogen Inc., CA, USA using Sanger sequencing. Sequences were trimmed by removing primer sequences from each end of the sequence with A Plasmid Editor software (ApE; Davis and Jorgenson 2022). The quality of sequences was visually assessed for distinct peaks at each nucleotide sequence. The trimmed sequences of the CO1 of eggs and larvae were compared to existing CO1 sequences in the NCBI nucleotide collection using BLAST, as well as additional sequences generated in this project from fin clips.

Statistics were performed using R software (R Team 2021). A frequency plot of egg diameter among unknown species was produced. Although other morphological features, such as number of oil globules, oil globule color, and pigmentation, were examined for eggs, data was omitted due to inconsistencies from egg damage. Distribution curves were produced using the kernel density smoothing function to visualize the probability of egg diameters of genetically confirmed species. Box plots identified outliers and median egg diameters. The Shapiro-Wilk test of normality was used to determine if egg diameter values among the subsample ($n = 31$) were normally distributed. After confirmation of the prerequisites of normality, a t-test was used to determine if there were differences ($p < 0.05$) in the means of egg diameters for genetically identified samples.

CHAPTER III

RESULTS

Mixed Species Egg Samples

The species identity of initial egg samples (n = 258) morphologically analyzed was unknown. The frequency plot of egg diameters was symmetrical and bell-shaped (Fig. 4). Egg diameters ranged from approximately 650 to 1084 µm, with a mean of 902 ± 81 µm (Fig. 4).

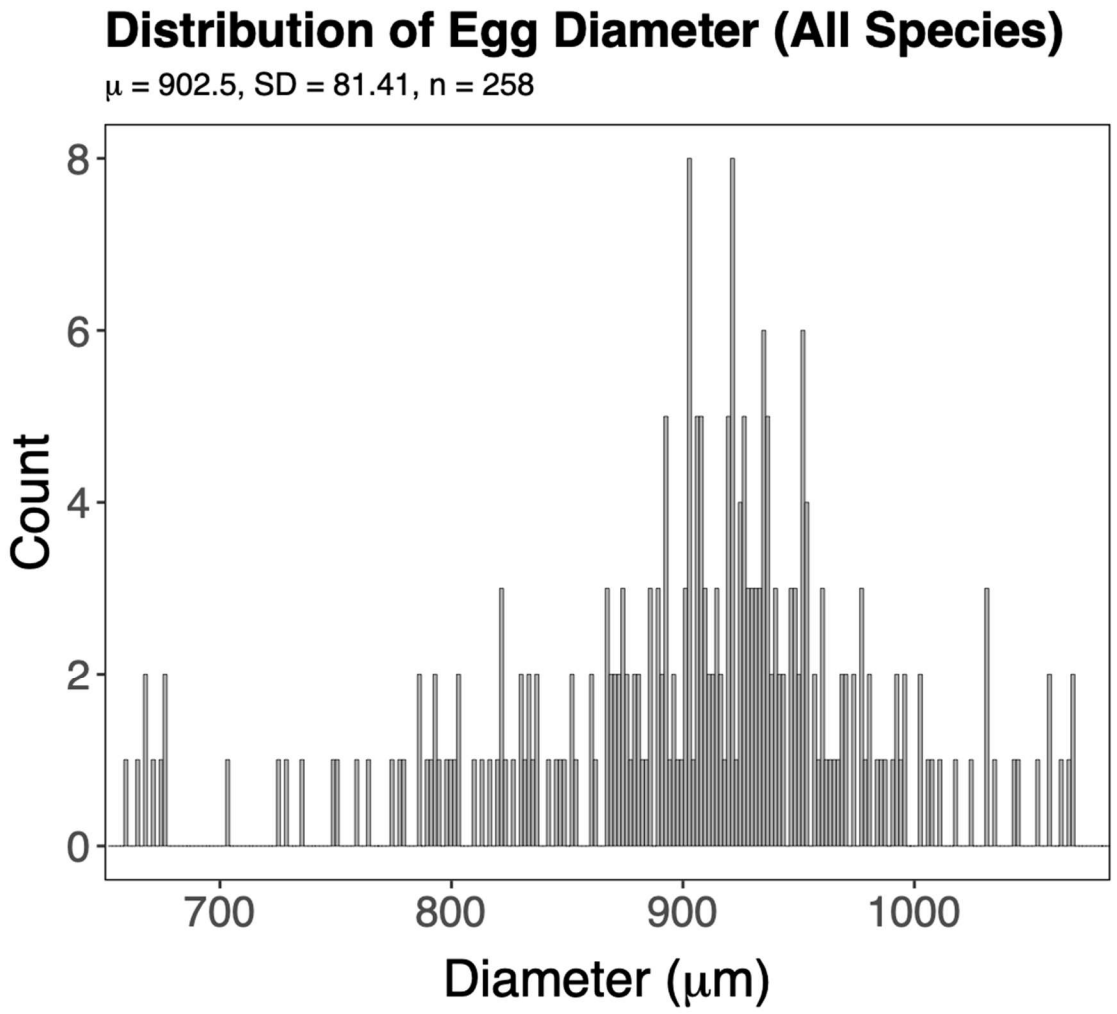

Figure 4: Egg diameter frequency (n = 258) from the mixed species Living Coral Reef exhibit at the Texas State Aquarium. Eggs ranged from 650 to 1084 µm, with a mean (± s.d.) of 902 ± 81 µm.

A small subsample (n = 31) of eggs was subsequently genetically identified to species. Egg diameter ranged from 670 to 930 µm for smallmouth grunt (*Brachygenys chyrsargyreum*)

and 909 to 1019 µm for cottonwick (*Haemulon melanurum*). Mean (± s.d.) egg diameters, 860 ± 45 µm for smallmouth grunt and 972 ± 30 µm for cottonwick, were significantly different ($p < 0.0001$) (Fig. 5). Distribution curves of egg diameter for the subsample had distinct peaks for each species with an overlap of outliers for the two species between 909 and 930 µm (Fig. 5).

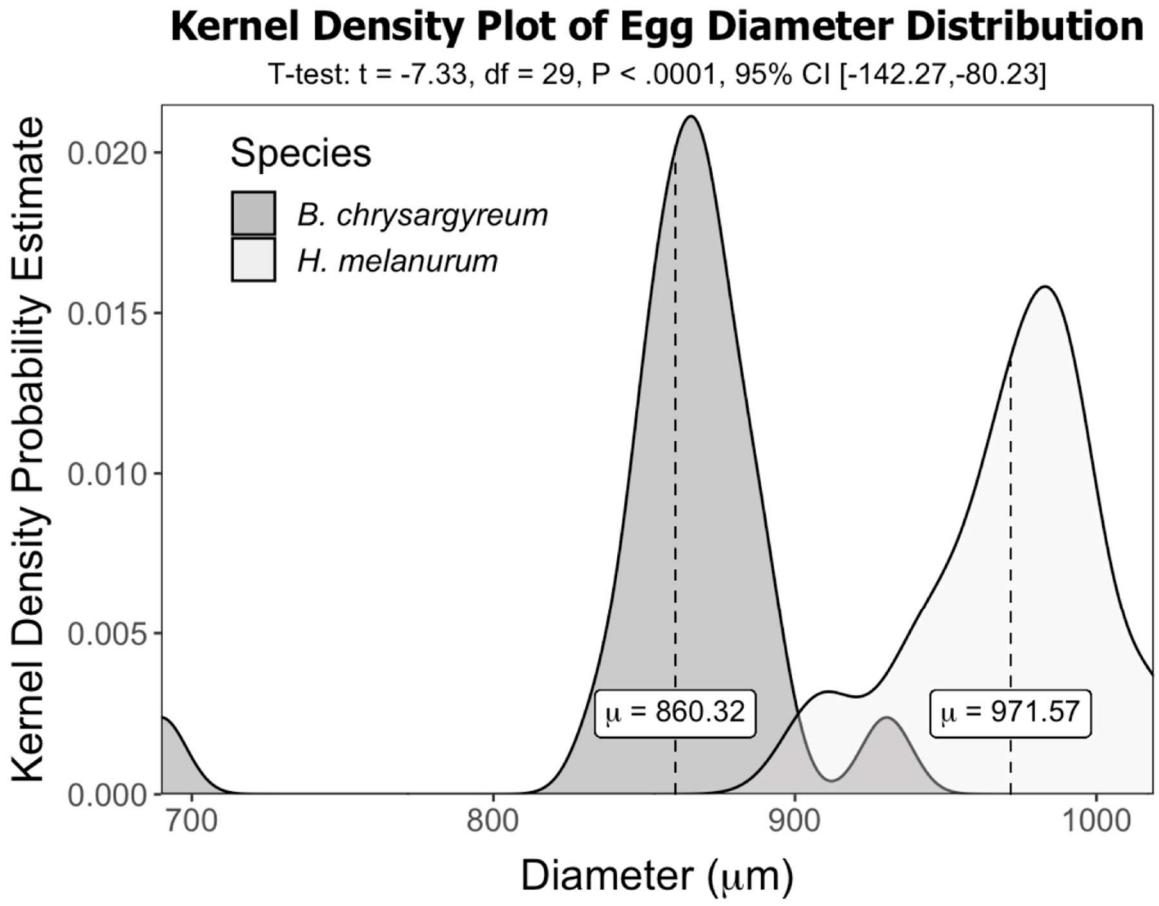

Figure 5: Kernel density distribution estimates of egg diameter counts by species. Mean (± s.d.) egg diameters were 860 ± 45 µm for smallmouth grunt (*Brachygenys chyrsargyreum*) (n = 20), and 972 ± 30 µm for cottonwick (*Haemulon melanurum*) (n = 11).

The boxplot of egg diameter for the subsample (Fig. 6) shows the interquartile ranges (IQR), consisting of the top 25% to lower 75% of values, as boxes for smallmouth grunt and cottonwick. Horizontal lines represent the median diameter for each species. The vertical lines below the boxes (IQRs) indicate the minimum egg diameter values to lower 25% of values for

each species, while vertical lines above boxes show the upper 25% to maximum egg diameter values. Sample outliers are shown as solid points (Fig. 6).

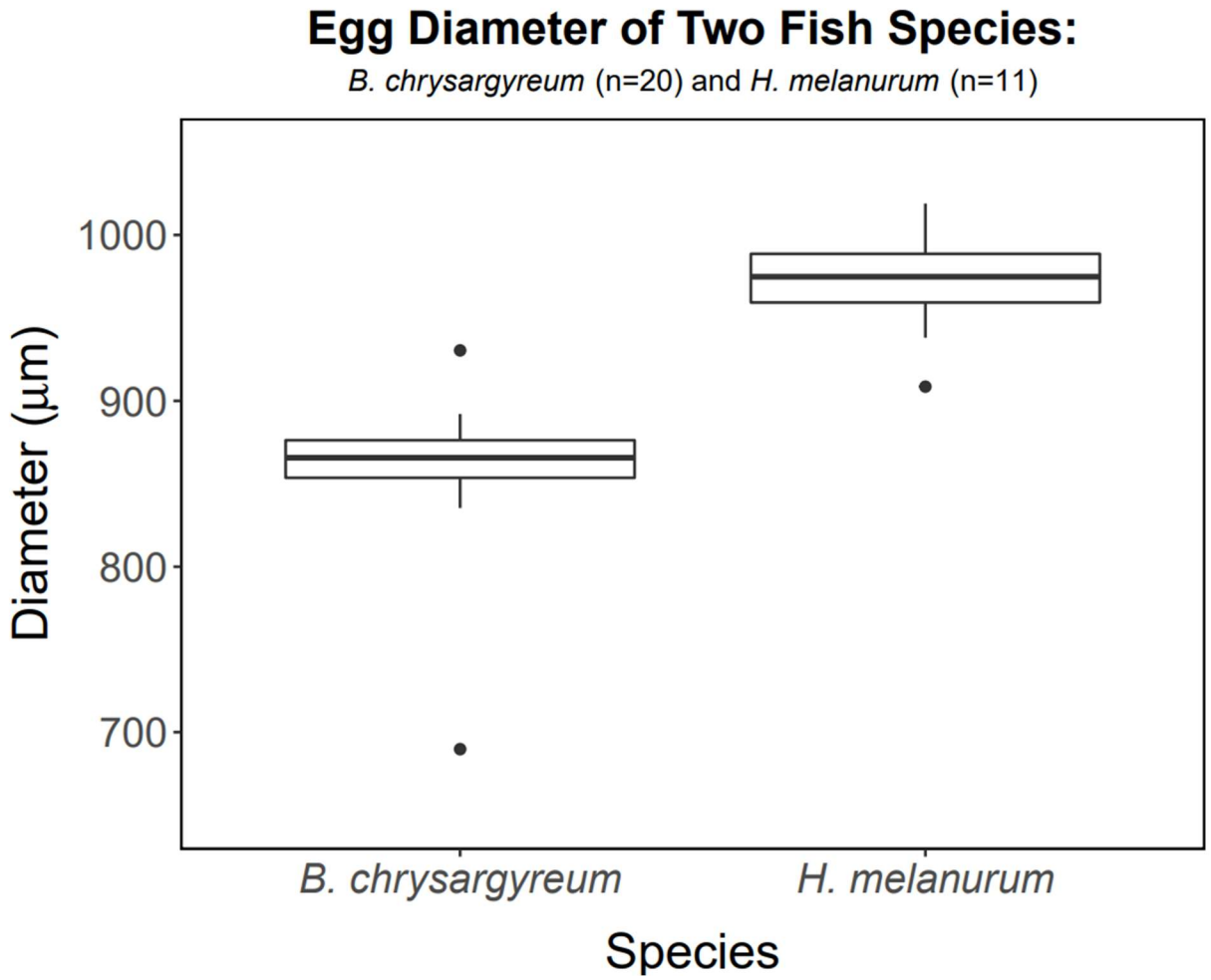

Figure 6: Boxplot of egg diameters for smallmouth grunt (*Brachygenys chyrsargyreum*) and cottonwick (*Haemulon melanurum*). Medians are represented by horizontal lines. The interquartile range (IQR), composed of the upper 25% to lower 75% of values, are shown as boxes for each species. Vertical lines display the minimum to lower 25% of values (bottom) and the upper 25% to maximum values (top). Egg diameter means, 860 ± 45 µm for smallmouth grunt and 972 ± 30 µm for cottonwick, were significantly different ($p < 0.0001$). Sample outliers are shown as solid points.

A single French angelfish (*P. paru*) egg was obtained in preliminary sampling in November 2019 and DNA was successfully sequenced. Although morphology was not examined for the single egg, this study is the first to document spawning of French angelfish the LCR exhibit at TSA during the natural 10-hour daylight photoperiod at 21°C and 30 ppt salinity.

Morphological features such as egg diameter, oil globule diameter, number of oil globules, and color of the oil globule were recorded for egg samples. The number of oil globules for all samples ranged from 0 to > 15 (Fig. B.16; Fig. B.17; Fig. B.18), with most eggs having a single, large oil globule (i.e., Fig. B.5; Fig. B.6). Oil globule color ranged from translucent (Fig. B.3) to dark yellow (Fig. B.5), and the oil globule sometimes migrated within the egg (Fig. B.10; Fig. B.11).

All eggs examined in the present study were translucent and spherical, with smooth outer chorions, homogenous yolk, and a narrow perivitelline space (Fig. B.1-B.18). Many of the eggs had misshapen chorion (Fig. B.3; Fig. B.12; Fig. B.13) and separation from the inner cytoplasm (Fig. B.9; Fig. B.10). Although distinct larval development was not observed, cellular differentiation was visible in the blastula (Fig. B.1). Ameboid cells were often distributed throughout the cytoplasm (Fig. B.1; Fig. B.2, Fig. B.9; Fig. B.15), while blastomeres were typically aggregated on one side of the egg (Fig. B.9; Fig. B.10; Fig. B.14). Dark round (Fig. B.2; Fig. B.6; Fig. B.8; Fig. B.17), and stellate cellular structures (Fig. B.4.; Fig. B.5; Fig. B.7), resembling melanophores, were present within some of the eggs of both species.

Bicolor Damselfish Larvae

All larval samples analyzed (n = 129) were bicolor damselfish (*Stegastes partitus*). Larvae were identified using morphology keys and were genetically confirmed (n = 19). The larvae had well-developed mouths, with no exogenous yolk sac, and were identified to species by characteristic pigmentation (Fig. 7). Dark, stellate melanophores were present on the forehead, anterior of the stomach, and ventral body (Fig. 7). Average total length of bicolor damselfish larvae was 1965 μm.

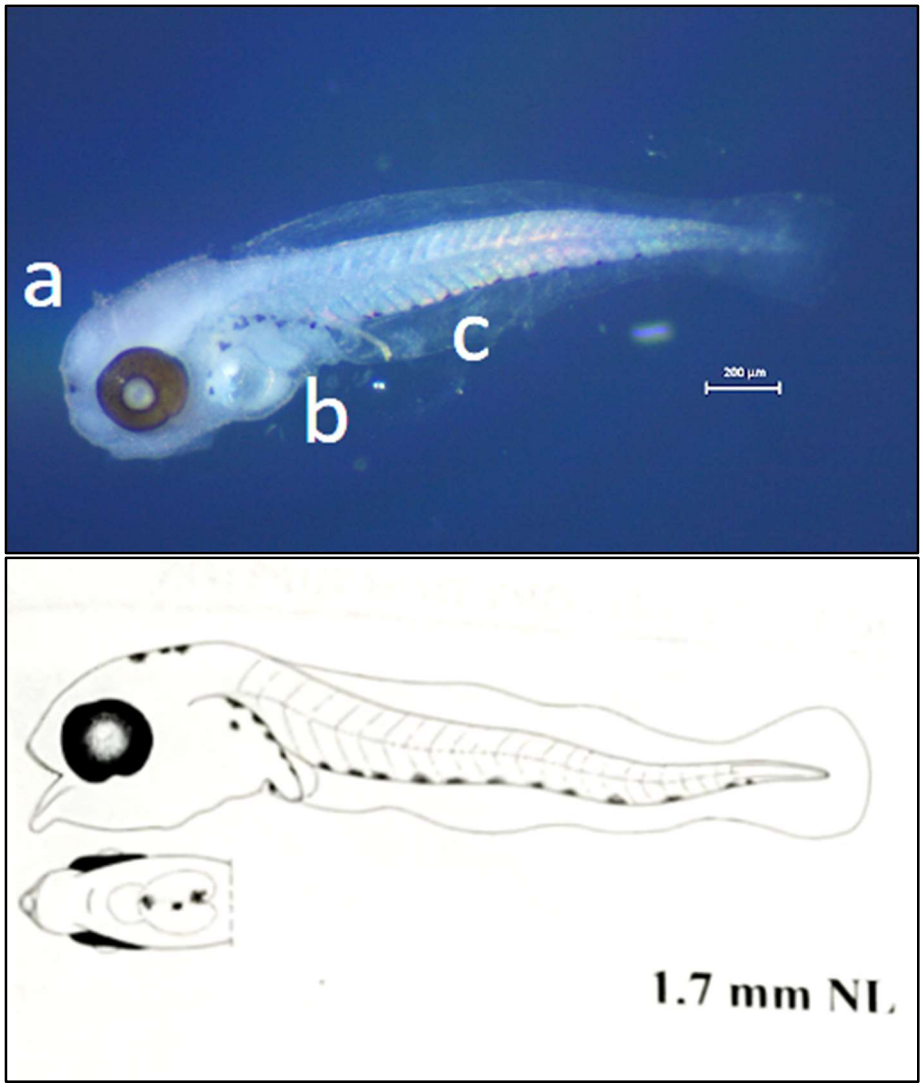

Figure 7: Photograph of bicolor damselfish (*Stegastes partitus*) larva 1 day post hatch (dph) compared to illustration of newly hatched *S. partitus*. Characteristic pigmentation is prominent on the a) forehead, or brain, b) anterior of stomach, and c) ventral body (above). Similar pigmentation pattern of newly hatched *S. partitus* (below) has been illustrated by J. Javench (Richards 2005).

CHAPTER IV

DISCUSSION

The present study documented distinguishable egg sizes for two species of grunts (*B. chrysargyreum* and *H. melanurum*), documented spawning of the French angelfish (*P. paru*), identified bicolor damselfish (*S. partitus*) larvae with existing literature, and verified methods of DNA barcoding with single eggs and larvae. This study is the first to report egg diameters for smallmouth grunt and cottonwick. Findings support the hypothesis that eggs and larvae of varied species may have distinguishing morphological features, such as egg diameter, that can be used for identification in aquariums with limitations. Egg diameter ranged from 670 to 930 µm (for smallmouth grunt and 909 to 1019 µm for cottonwick, and mean egg diameters (860 ± 45 µm and 972 ± 30 µm) were significantly different ($p < 0.0001$) for the two species.

Overlap between egg diameters of the two species makes it impossible to identify eggs within this range (909 and 930 µm) to species at this stage. Despite limitations with sampling, preservation, and sequencing, this research contributes to the limited knowledge of early life history of reef fishes. The egg diameter ranges reported in this study can contribute towards a catalog of eggs and larvae for larval culture in aquariums.

Egg Morphology for Identification

The present study determined that egg size demonstrated some potential for differentiating the two species of grunt from the Living Coral Reef (LCR) exhibit at the Texas State Aquarium (TSA). The mean egg diameters of smallmouth grunt and cottonwick were statistically different (860 ± 45 µm and 972 ± 30 µm, respectively), supporting the hypothesis that eggs of different species exhibit different morphological features. Eggs ranged from 690 to 930 µm for smallmouth and 909 to 1019 µm for cottonwick. Similar egg diameters ranges were

seen in the mixed samples (650 to 1084 µm). Although outliers overlapped between 908 and 930 µm for the two species, egg diameters of smallmouth grunt ranged from 835 to 892 µm, while egg diameters of cottonwick ranged from 938 to 1019 µm without outliers. In this study, eggs below 892 µm could be distinguished as smallmouth grunt, while eggs above 938 µm could be distinguished as cottonwick. However, overall, egg morphology proved to be of limited power to distinguish species from mixed exhibits without DNA barcoding. Time spent measuring eggs could be more efficiently used to grow out to larvae to facilitate identification in the future.

Smallmouth grunt and cottonwick have been previously reared from egg by Fish Eye Aquaculture.This study is the first to report and compare egg diameters for the two species. The findings in the present study are consistent with other studies, documenting year-round spawning of French grunt (*H. flavolineatum*), peaking in warm months (Courtenay 1961; Hauville et al. 2017; Maurer et al. 2020). While the mean egg diameter of smallmouth grunt and cottonwick differed significantly, egg diameter alone cannot be definitively used to identify to species as a wide range of egg diameters exists among unknown species. Outliers in egg diameter values imply that, although mean egg diameter may differ significantly for each species, the variation in egg diameter within individuals of the same species makes it virtually impossible to distinguish species by egg size alone.

Hauville et al. (2017) and Maurer et al. (2020) reported minimal variation in egg diameter among French grunts. Both studies describe eggs of French grunt as transparent, spherical, pelagic, with a yellowish oil globule, and larval development present. Hauville et al. (2017) reported eggs measuring 0.96 ± 0.03 mm, hatching within 20 h post fertilization. Maurer et al. (2020) documented a mean egg diameter of 0.95 ± 0.006 mm. Similarly, cottonwick eggs documented in the present study were transparent, spherical, and measured 0.97 ± 0.03 mm. In

opposition, larval development was not distinguishable in eggs examined at 15 h post fertilization for the present study, as eggs appeared to be in the blastula stage. Extra eggs and larvae collected on the last day of sampling were placed in a glass beaker (covered in black paper) for observation by aquarists at TSA. Although observations were beyond the scope of the present study, eggs hatched within 1-2 days after collection. All eggs and larvae expired within 3 days of collection, likely due to starvation from inappropriately sized *Brachionus sp.* rotifers and *Artemia sp.* nauplii, and cross contamination with other systems.

The practicality of utilizing other morphological features examined during this study was inconclusive. The open-ocean egg identification study by Kawakami et al. (2010) divided captured eggs into types based on morphological characteristics including egg shape, egg diameter, structure of the chorion, presence or absence of oil globule, size and color of oil globule, pigmentation, yolk segmentation, and width of the perivitelline space. Similarly, the study by Callan et al. (2012) examined flame angelfish (*C. loriculus*) eggs collected daily, at approximately 15 h post fertilization, characterizing eggs as infertile, fertile-inviable, and viable. The study measured egg diameter along the body axis, oil globule diameter, length at hatch, and length at 3 days post hatch (dph) (Callan et al. 2012). Hatch rate and survival to 3 dph was calculated and results demonstrated little change in egg measurements. However, embryo viability was significantly higher for eggs from parents fed raw diets and diets rich in highly unsaturated fatty acids (HUFAs) (Callan et al. 2012; Callan et al. 2014).

Although the number of oil globules, oil globule diameter, oil globule color, embryo total length, and embryo coloration and pigmentation were examined in this study, these features were difficult to distinguish so early in the pre-larval stage (Matarese and Sandknop 1984; Moser 1996; Alshuth et al. 1998; Richards 2005). The number and color of oil globules was variable

among the two species in this study, possibly due to damage. Observations of damage are consistent with the descriptions of mechanical damage in early-stage eggs by Wourms (1972). The developmental stage of eggs in this study were consistent with the explanation by Ahlstrom and Moser (1980), where deep blastomeres consolidate in a mass, then migrate outward as amoeboid cells.

Many of the eggs analyzed in the present study were visibly damaged, having features consistent with overripening (Tucker 1998). Some eggs had misshapen or degenerated chorion, "clumped" cytoplasm, and dark round and stellate structures resembling melanophores. Sample damage could potentially be contributed to collection techniques. Eggs were pipetted from beaker to watch glass without supplemental aeration prior to preservation. The crushed outer egg shape and separation of the inner contents from the chorion could have been caused by mechanical damage during and after collection (Hemple 1979).

The advantages of DMSO for genetic analysis have been demonstrated over ethanol or formalin (Dawson et al. 1998; Kawakami et al. 2010; Gordeeva et al. 2019). Unfortunately, the effects of DMSO and other preservation methods on the morphology of marine fish eggs remains undocumented. Fost et al. (2020) found that dry ice was effective for genetic and morphological preservation of fish embryo, however, the effects of different preservation methods on the morphology of eggs should be further examined. The quality of eggs can also be influenced by maternal factors. Callan et al. (2012) found that mean fertilization rates and egg viability was significantly greater in flame angelfish (*C. loriculus*) when broodstock were fed a raw diet versus a formulated feed. Congruently, Callan et al. (2014) found significant increases in fecundity, fertilization rates, and embryo viability when brookstock were fed diets high in HUFAs.

Sequencing challenges limited the number of eggs analyzed during the present. Although a total of 258 eggs and 129 larvae were morphologically analyzed and prepared for DNA barcoding, sequences were only obtained for a single egg and 8 larvae. Limited PCR amplification was likely due to degradation of DNA from prolonged refrigeration post extraction. The extracted DNA was left at 12°C for several months during sample preparation in 2020. Early successes in sequencing preliminary samples preserved in seawater resulted in sequences for a single French angelfish egg and 11 bicolor damselfish larvae. Excess salts from DMSO in the extraction processes could have later inhibited PCR, and not fully macerating the outer shell of the eggs could have limited the amount of DNA extracted (Kawakami et al. 2010). Protocols were adjusted because of these challenges and genetic information was successfully obtained from a subsample (n = 31) of eggs collected during the experimental period. However, the size of the subsample was limited by the number of eggs from the original collection dates.

Although the present study did not examine the larval stages of smallmouth grunt and cottonwick, previous studies have described French grunt larvae (Illustration by Lindeman 1986; Hauville et al. 2017). Newly hatched larvae measured 2.69 ± 0.05 mm notochord length (NL) and had faint midventral and middorsal internal blotches, no pigment on snout or lower jaw, melanophores scattered on anterior margin of the dorsal (Hauville et al. 2017). Additionally, the University of Florida's Tropical Aquaculture Lab (UFTAL) measured embryo diameter, as well as standard length (SL) and total length (TL) from hatch to 54 dph during the development of Pacific blue tang (*P. hepatus*) (DiMaggio et al. 2017). Documenting these features, embryo development in eggs at different stages, growing out to hatch, and larval rearing will give a more comprehensive picture of early life history, making sorting by species more practical in a dynamic setting (Callan et al. 2014; Cassiano et al. 2015; Olivotto et al. 2017).

Temporal collection throughout the year would allow for a better understanding of spawning conditions for a variety of species. A single French angelfish egg was obtained from the LCR exhibit in preliminary sampling during November 2019 and DNA was successfully sequenced. Although morphology was not examined for the single egg, this is the first documentation of French angelfish spawning in the LCR exhibit, during the natural 10.5-hour daylight photoperiod, at 21°C and 30 ppt salinity. These findings are consistent with previous documentation of the reproduction of French angelfish and other large angelfishes spawning in tropical winter (Thresher 1980; Feitosa et al. 2015; Holt et al. 2017). Leu et al. (2009) provided the first description of spawning of semicircle angelfish (*P. semicirculatus*) in captivity, documenting buoyant, spherical, transparent eggs with a mean diameter of 0.61 ± 0.03 mm. Embryo development for the species was observed between 18 and 21 h at 21.6 C (Leu et al. 2009). Newly hatched larvae measured 1.35 ± 0.02 mm TL and had 27 (12 ± 15) myomeres (Leu et al. 2009). The oil globule was in the ventroposterior area of yolk sac, with total yolk absorption within 3 dph (Leu et al. 2009). Olivotto et al. (2006) similarly documented development of lemonpeel angelfish (*C. flavissimu*s), outlining protocols for live feed concentrations and schedules. Similar larviculture techniques were utilized by Baensch and Tamaru (2009) in reporting the spawning and development of the rare blue Mauritius angelfish (*Centropyge debelius*). Replication of protocols contributed to subsequent success in the captive hybridization of the two geographically isolated pygmy angelfish species, Fisher's angelfish (*Centropyge fisheri*) and resplendent angelfish (*Centropyge resplendens*), by Baensch and Tamaru (2010).

Mendonca et al. (2020) documented the embryonic and early development of the bicolor angelfish (*Centropyge bicolor*) and coral beauty angelfish (*Centropyge bispinosa*). The three

popular species, rock beauty (*Holacanthus tricolor*), queen angelfish (*Holacanthus ciliaris*), and French angelfish, are among the top candidates at risk for overexploitation in ornamental fisheries (Hauville et al. 2017; Dee et al. 2019; Holcombe et al. 2022). These species have a high market demand and commercial aquaculture potential, but rearing protocols have not been established for these species. Successful improvements in feeding methods, such as those for the commercial-scale culture of yellow tang (*Z. flavescens*), can be adapted for large-scale larval rearing in zoos and aquariums (Olivotto et al. 2011; St. Leger and Violetta 2017; Callan et al. 2018). Replicating these studies can be advantageous in developing larval rearing protocols for French angelfish and other large angelfishes.

Larval Identification by Morphology

Bicolor damselfish larvae in this study were identified using morphology keys, and confirmed with DNA barcoding, supporting the hypothesis that early-stage larvae can be identified using distinct features. The present study utilized total length (TL) and pigmentation to identify larvae (Alshuth et al. 1998; Richards 2005; Leu et al. 2009). Although bicolor damselfish were the only larvae collected during the sampling period, this study confirms previous descriptions of bicolor damselfish larvae. Larvae (n = 129), approximately 1 dph, measured 2.0 mm TL and had characteristic forehead, stomach and ventral stellate pigmentation (Emery 1968; Robertson et al. 1988; Sponaugle and Cowen 1996; Paris-Limouzy 2001). A subsample of larvae (n = 19) was used for genetic analysis to confirm species identity.

Eggs were not identified for bicolor damselfish in the present study; however, others documented single layer demersal egg clutches (Courtenay 1961; Munro et al. 1973). Year-round spawning occurs in the early evening (Hildebrand and Cable 1930) and peaks in spring and summer (Paris-Limouzy et al. 2005). Eggs are bright white and change to dark green during the

four-day incubation period (Courtenay 1961; Emery 1968; Munro et al. 1973; Lindeman 1986; Robertson et al. 1988; Sponaugle and Cowen 1996; Lindeman 1997; Paris-Limouzy 2001). Larvae collected in this study likely hatched overnight in the LCR exhibit and were pulled into the egg and larva collector while the skimmers to the exhibit were turned off. Sme (2012) similarly identified bicolor damselfish and white grunt (*H. plumerii*) using morphological and genetic analysis.

Pomacanthidae is one of the most imported families in the U.S. and the life history and culture has been documented (Olivotto et al. 2006; Rhyne et al. 2017). Standardized production methods have been outlined for pomacanthids, including numerous species of clownfish, three spot damsel (*Dascyllus trimaculatus*), Humbug damsel (*D. aruanus*), and Caerulean damsel (*Pomacentrus caeruleus*) (Gopakumar et al. 2009). Gopakumar et al. (2009) also made developments in protocols for blue green damsel (*Chromis viridis*), yellowtail damsel (*Neopomacentrus nemurus*) and cloudy damsel (*Dascyllus carneus*). Anzeer et al. (2019) documented the development and larval rearing of cloudy damsel (*D. carneus*). Compact tank designs for larval culture research were made available through literature and implemented with newly hatched blue devil damselfish (*Chrysiptera cyanea*) (Moorehead 2015).

Successful breeding programs for other organisms in zoos and aquariums, such as the Jellyfish Culture Facility at Monterey Bay Aquarium and the New England Aquarium Jellyfish Culturing Facility, can be modelled and fitted for rearing larval fish (Hart and Warmolts 2008). TSA and other AZA accredited facilities have implemented culture of jellyfish and seahorses, with considerable popularity among guests (Hart and Warmolts 2008; Cassiano et al. 2015; Olivotto et al. 2017; Pouli et al. 2019). The findings from the present study can be utilized with established larval rearing protocols, such as those for French grunt (Hauville et al. 2017), Pacific

blue tang (DiMaggio et al. 2017), semicircle angelfish (Leu et al. 2009), flame angelfish (Callan et al. 2014), and damselfishes (Gopakumar et al. 2009; Rajasekar 2009; Anzeer et al. 2019; Holcombe et al. 2022). Public aquariums offer the unique opportunity of conservation impact through education on sustainable sourcing and aquaculture exhibits, partnerships with universities and other organizations (St. Leger and Violetta 2017), in addition to access to materials such as tanks and broodstock (Buckley et al. 2005; Olivotto et al. 2011; Tlusy et al. 2012; Tlusty et al. 2017; St. Leger and Violetta 2017).

Conclusions

The present study provides a verification of methods for DNA barcoding and morphological characterization of eggs and larvae in public aquariums. Egg diameter, with genetic verification, may be a practical tool for sorting eggs in public aquariums. This study documented the eggs of smallmouth grunt and cottonwick, demonstrating that egg size comparisons may be used to differentiate these two species. Additionally, the unique pigmentation patterns of larval fish, as early as 1 dph, allow for species identification with existing morphological keys. Efficient sorting of eggs and larvae in public aquariums may reduce the resources needed to create and implement species-specific larval rearing protocols. These findings contribute towards a comprehensive identification key for aquarium fishes by documenting unique characteristics of egg and early larval stages.

The present study provides a path towards potential larval rearing of bicolor damselfish, smallmouth grunt, cottonwick, and French angelfish at TSA. Examining eggs and larvae throughout development and modelling successful culture protocols will increase the capacity of public aquariums to support sustainable sources of marine ornamentals. Continued partnerships with organizations like RTC and other ACA accredited facilities will maximize conservation

efforts through the exchange of vital information and techniques. Creating and implementing Species Survival Plan (SSP) programs and Institutional Collection Plans (ICPs) will aid in focused conservation efforts aligning with the goals and missions of public aquariums. The ecological value of desirable aquarium species must be considered when assessing the costs associated with raising larvae in comparison to the price of wild caught individuals. Larval culture in aquariums can provide the opportunity to promote conservation through new and updated exhibits. Featuring and engaging people with captive bred fishes can lead to increased public interest and awareness on the value of marine ornamentals in reef ecosystems.

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APPENDIX A

LIFE SUPPORT DIAGRAM

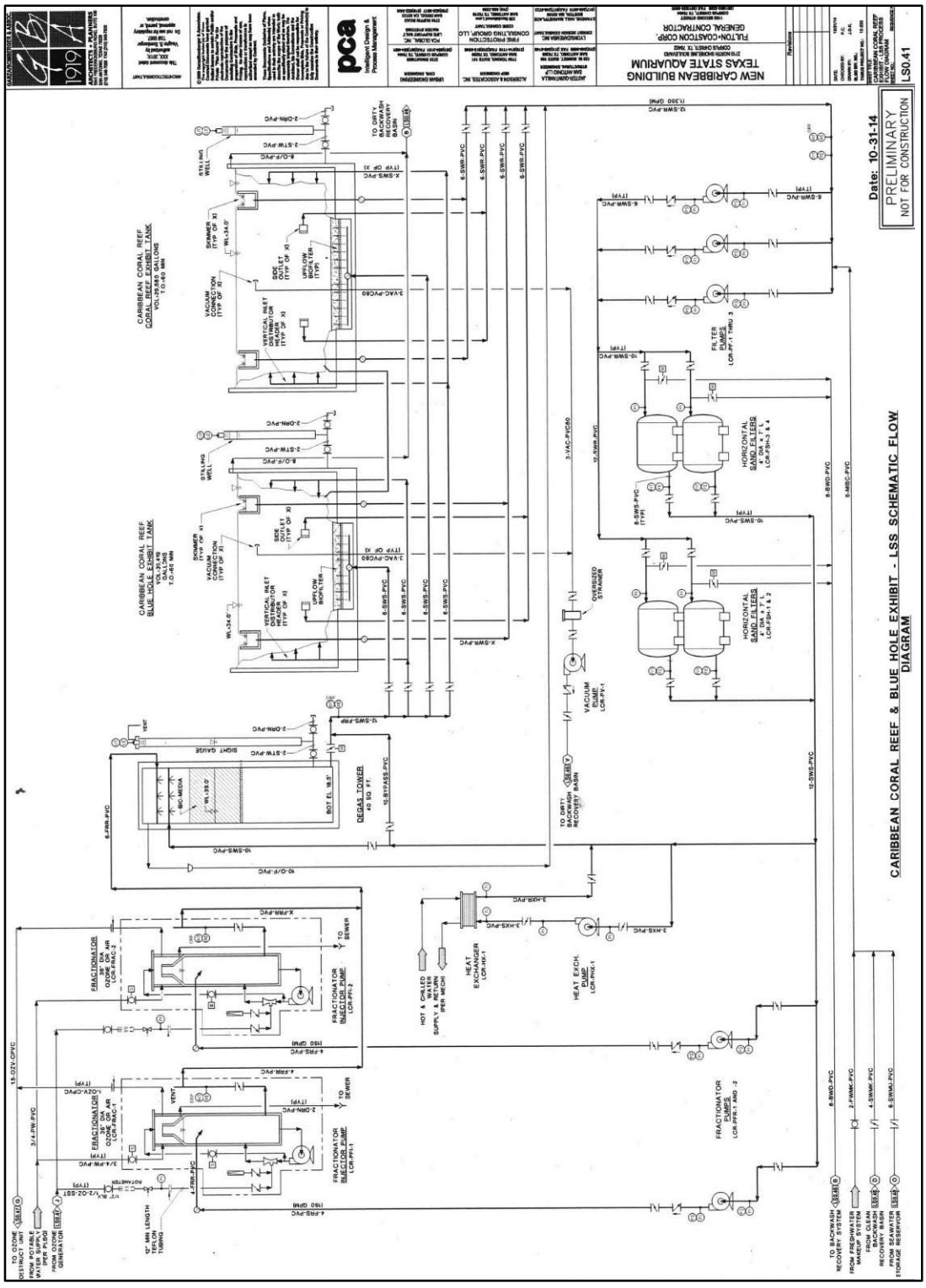

Figure A1: Life support schematic of the Living Coral Reef exhibit at the Texas State Aquarium.

APPENDIX B

EGGS FROM THE LIVING CORAL REEF EXHIBIT

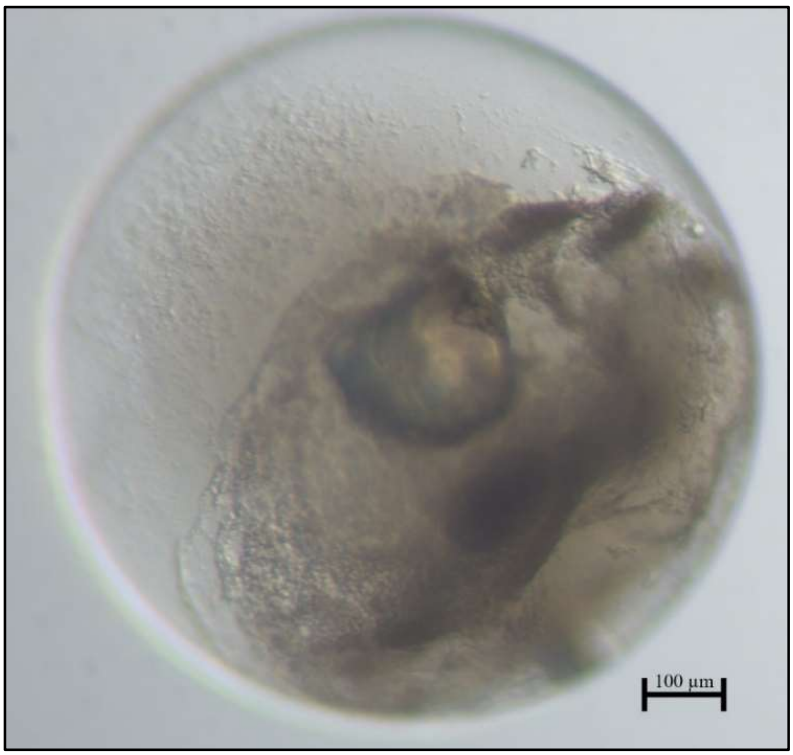

Figure B.1: Damaged smallmouth grunt (*B. chyrsargyreum*) egg with (outer to inner) separation of the enveloping layer from the chorion, irregularly-shaped, light-yellow oil globule, clumped cytoplasm, and aggregation of blastomeres.

Figure B.2: Damaged smallmouth grunt (*B. chyrsargyreum*) egg with clumped cytoplasm, and round structure on the light-yellow oil globule.

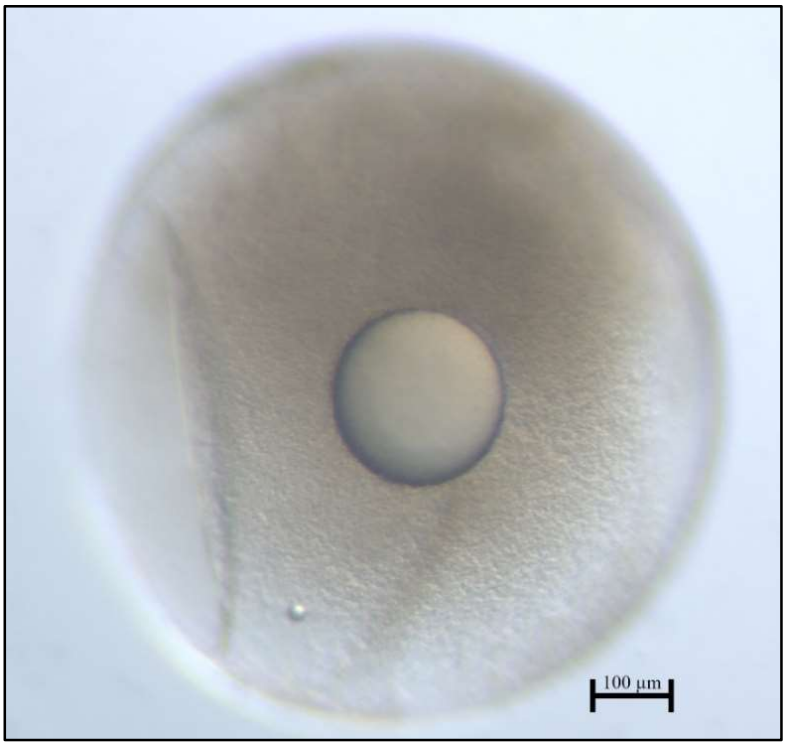

Figure B.3: Damaged smallmouth grunt (*B. chyrsargyreum*) egg with crushed outer chorion, light-yellow oil globule, and small additional oil globule.

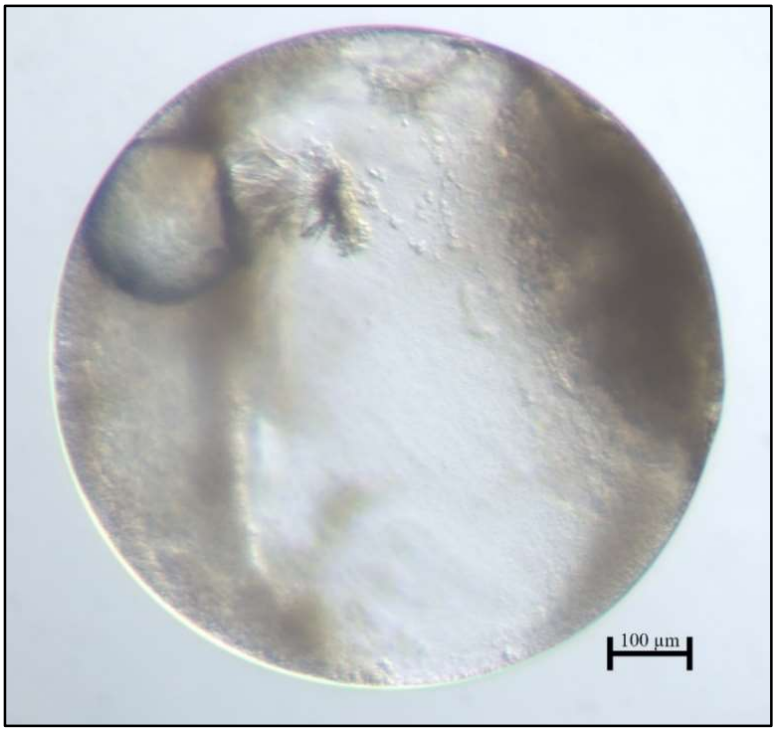

Figure B.4: Smallmouth grunt (*B. chyrsargyreum*) egg with separation from the chorion, clumped cytoplasm, cellular aggregation, irregularly-shaped and translucent oil globule, and stellate structure.

Figure B.5: Smallmouth grunt (*B. chyrsargyreum*) egg with clumped cytoplasm, yellow oil globule, and stellate structure.

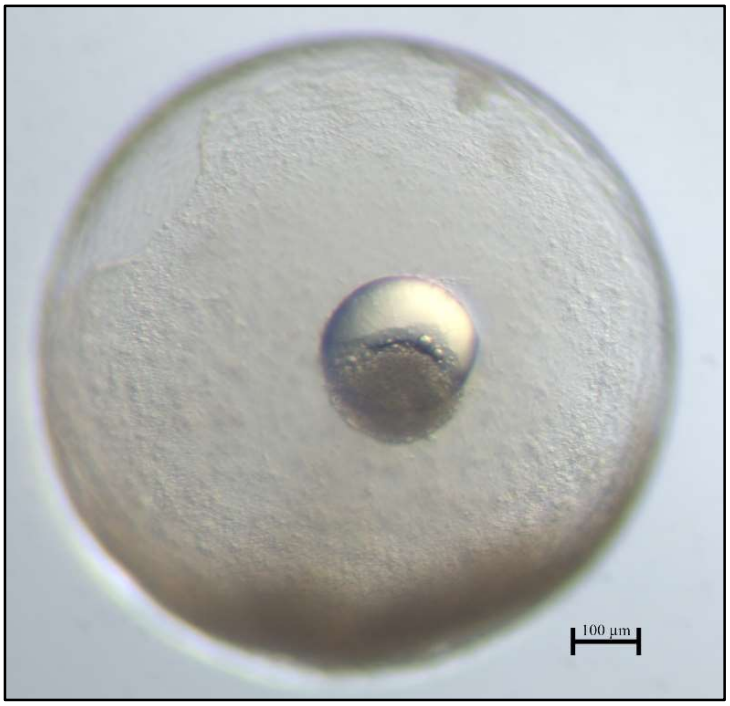

Figure B.6: Damaged cottonwick (*H. melanurum*) egg with separation of the inner yolk layer from the chorion, clumped cytoplasm, and round structure on the translucent to light-yellow oil globule.

Figure B.7: Damaged smallmouth grunt (*B. chyrsargyreum*) egg with crushed outer chorion, separation of the inner enveloping layer from the chorion, clumped cytoplasm, stellate structure, light-yellow oil globule, and additional small oil globules.

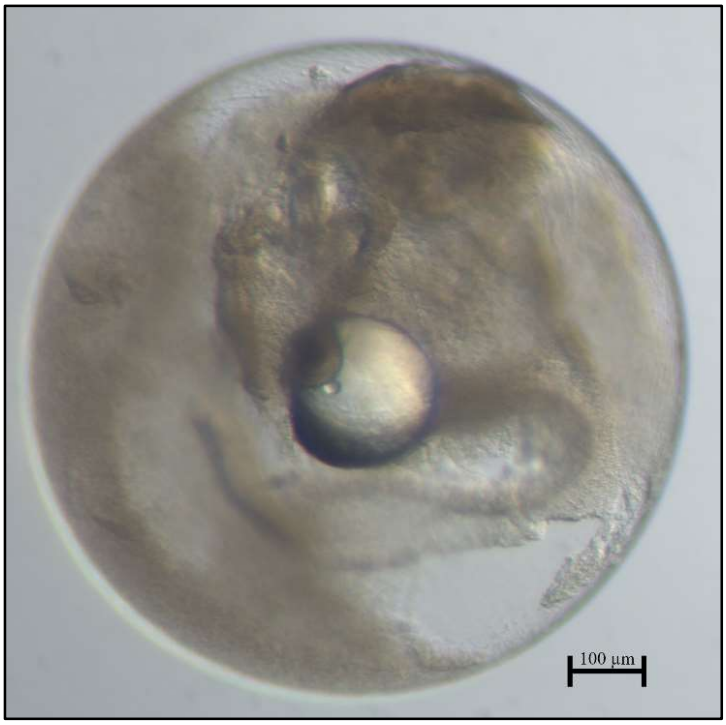

Figure B.8: Damaged smallmouth grunt (*B. chyrsargyreum*) egg with separation of the enveloping layer from the chorion, clumped cytoplasm, cellular aggregation, dark structure on the translucent oil globule, and small additional oil globule.

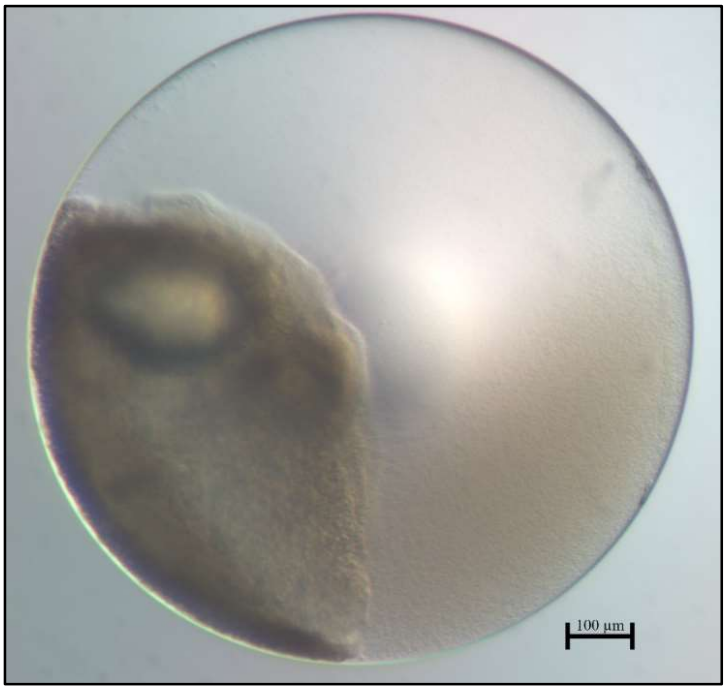

Figure B.9: Damaged cottonwick (*H. melanurum*) egg with distinct separation of the enveloping layer from the chorion, clumped cytoplasm, aggregate of cells, and irregularly-shaped, light-yellow oil globule.

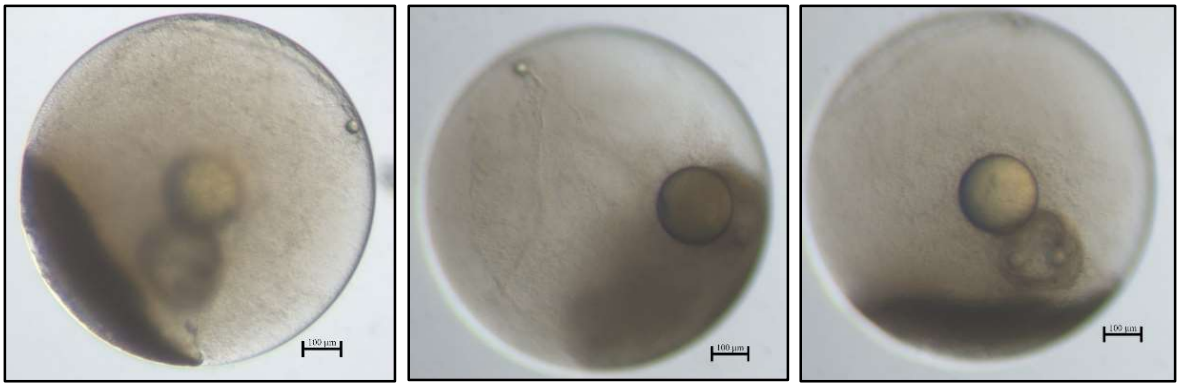

Figure B.10: Damaged cottonwick (*H. melanurum*) egg with separation from the chorion, clumped cytoplasm, cellular aggregate, migration of the yellow oil globule within the egg, and addional small oil globules.

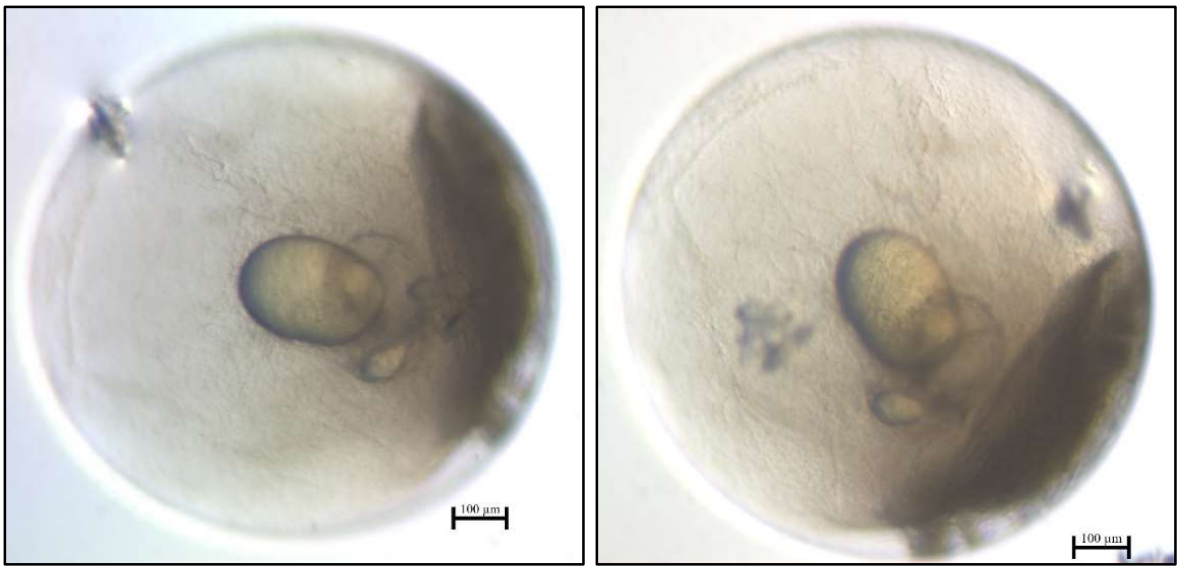

Figure B.11: Damaged cottonwick (*H. melanurum*) egg with separation from the chorion, clumped cytoplasm, cellular aggregate, migration of the yellow oil globule, and small additional oil globule.

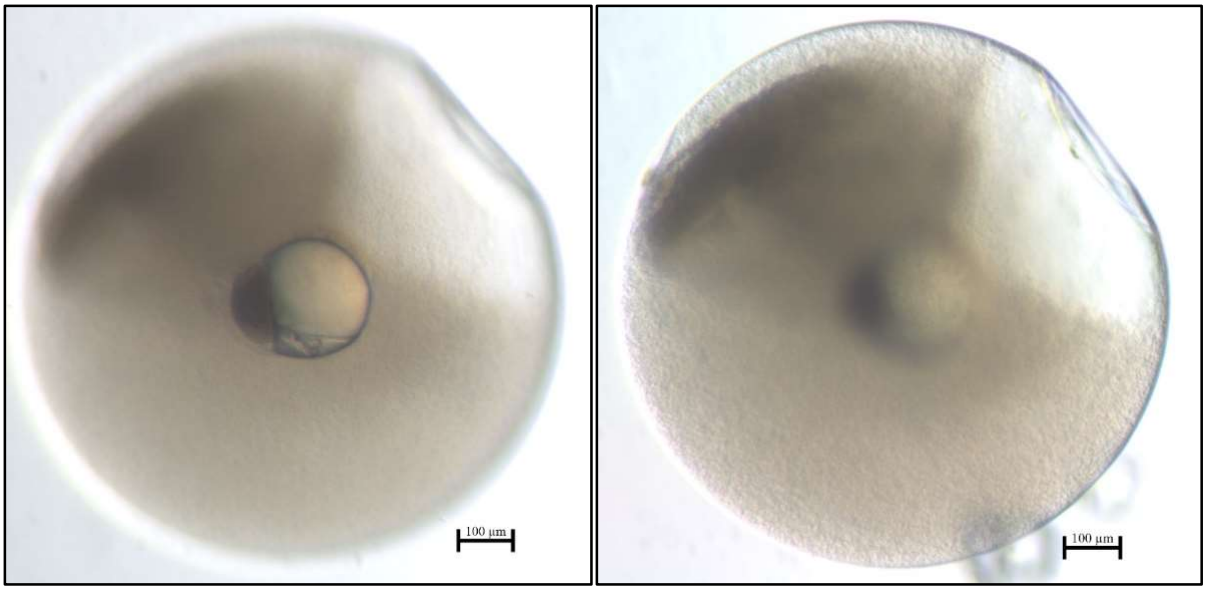

Figure B.12: Damaged smallmouth grunt (*B. chyrsargyreum*) egg with distinct, crushed outer chorion, separation from the chorion, cellular aggregation, and round structure on the light-yellow oil globule.

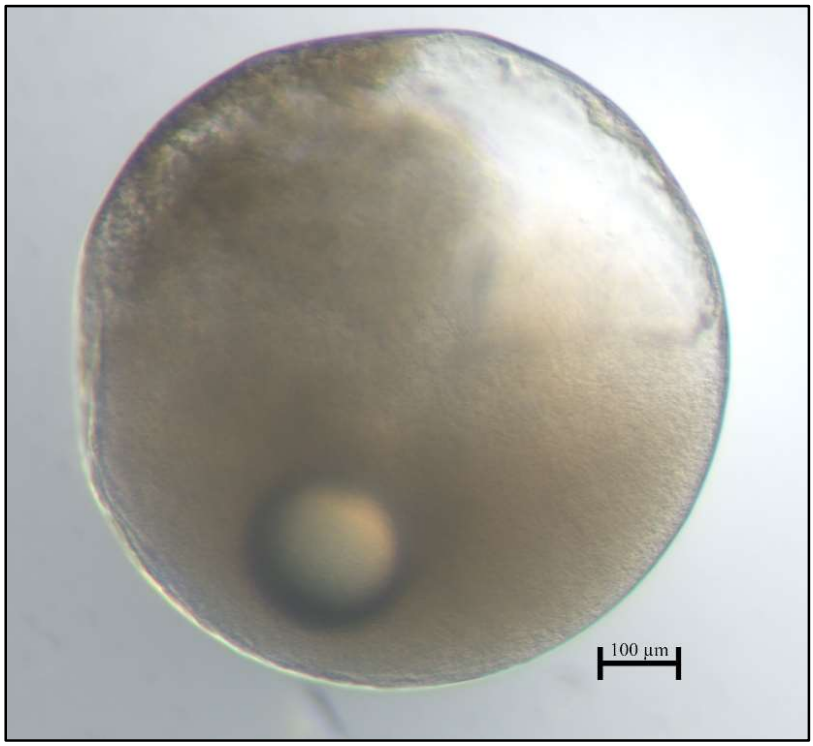

Figure B.13: Damaged cottonwick (*H. melanurum*) egg with crushed outer chorion, separation from the chorion, clumped cytoplasm, cellular aggregation, and light-yellow oil globule.

Figure B.14: Damaged smallmouth grunt (*B. chyrsargyreum*) egg with separation from the chorion, clumped cytoplasm, cellular aggregation, and dark, round structure on the light-yellow oil globule.

Figure B.15: Cottonwick (*H. melanurum*) egg with separation from the chorion, clumped cytoplasm, cellular aggregation, and dark, stellate structure on the light-yellow oil globule.

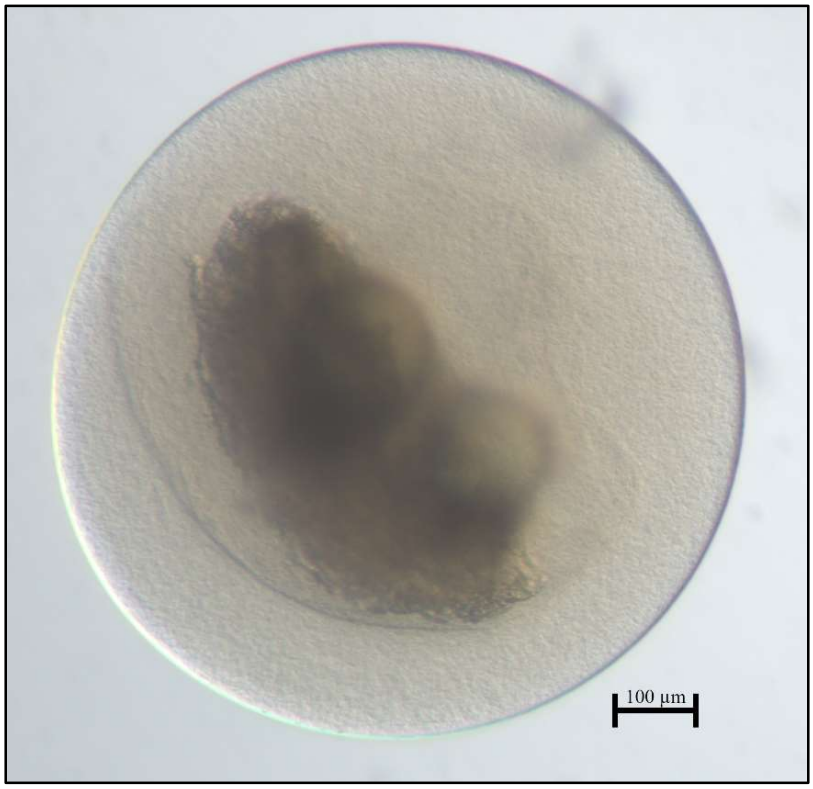

Figure B.16: Smallmouth grunt (*B. chyrsargyreum*) egg with crushed outer chorion, clumped cytoplasm, cellular aggregation, and two light-yellow oil globules.

Figure B.17: Cottonwick (*H. melanurum*) egg with separation from the chorion, clumped cytoplasm, cellular aggregation, and dark, round structure on one of two yellow oil globules.

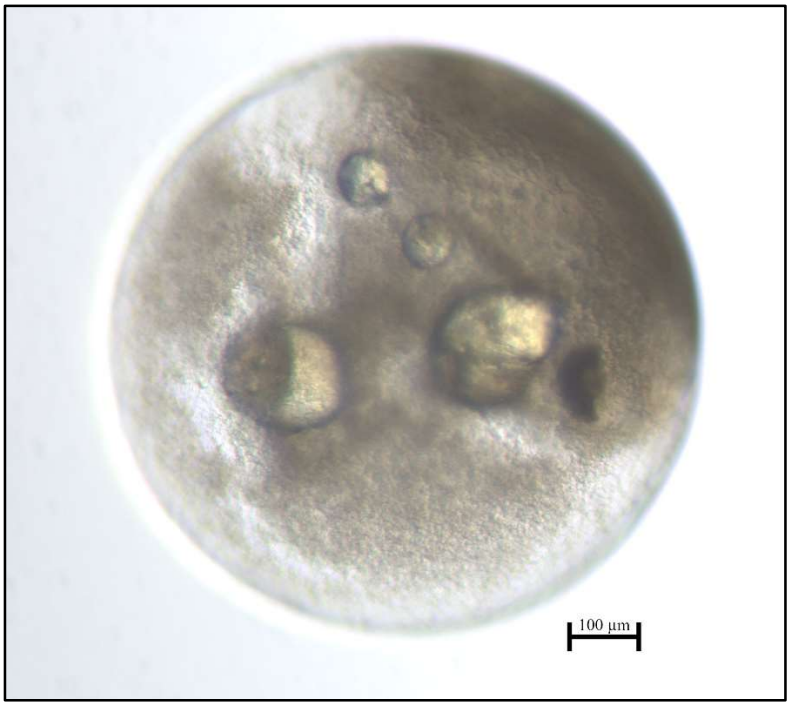

Figure B.18: Smallmouth grunt (*B. chyrsargyreum*) egg with separation from the chorion, clumped cytoplasm, cellular aggregation, and four light-yellow oil globules, with one dark spot on the oil globule.