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Assessing Monkeyface Prickleback Cebidichthys Violaceus as an Emerging Aquaculture Species in California

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ASSESSING MONKEYFACE PRICKLEBACK *CEBIDICHTHYS VIOLACEUS* AS AN EMERGING AQUACULTURE SPECIES IN CALIFORNIA

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Matthew Hoehn

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ASSESSING MONKEYFACE PRICKLEBACK *CEBIDICHTHYS VIOLACEUS*, AS AN EMERGING AQUACULTURE SPECIES IN CALIFORNIA

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DEDICATION

For, family, friends, fish, and fun

EPIGRAM

Year after year
On a Monkey's face
A monkey face
-Basho (Matsuo Munefusa, 1644-1694), haiku's first great practitioner.

ABSTRACT

Assessing monkeyface prickleback *Cebidichthys violaceus* as an
emerging aquaculture species in California
by
Matthew Hoehn
Master of Science (Marine Science) in Moss Landing Marine Labs

California State University Monterey Bay, 2023

In the context of global seafood demand and the pressing need for sustainable aquaculture practices, identifying and developing new species for aquaculture is of paramount importance. This study provides a comprehensive analysis of the reproductive behavior and embryological development of the monkeyface prickleback (*Cebidichthys violaceus*), an emerging aquaculture species in California. The species exhibits several desirable traits for aquaculture, including herbivory, sedentary behavior, high tolerance to environmental extremes, high fillet-to-carcass ratios, and product similarity to high-value, less sustainable fishes such as unagi. Since very little reproductive behavior was available at the start of these studies the experiments were designed on a broad basis. This research therefore intends to give a general description of the reproductive behavior of the monkeyface prickleback. This study employed geometric morphometrics, visual assessment, and ultrasound techniques to identify sexual dimorphisms, optimizing broodstock management. This study's results revealed significant differences in head shape (Procrustes ANOVA, $F_{46,4508} = 25.76$, p-value < 0.0001) and supraorbital crest shapes (Procrustes ANOVA, $F_{30,2940} = 5.96$, p-value < 0.001) and eye placements between sexes (Procrustes ANOVA, $F_{8,392} = 49.04$, p-value < 0.0001), with ultrasound proving 96.7% accurate for sex identification. For all landmarks sex influenced shape change (ANCOVA, sex: $F_{1,95} = 344.53$, p value $< 2.2e^{-16}$) and the interaction between length and sex influenced shape change (ANCOVA length:sex $F_{1,94} = 24.26$ p value $< 3.59e^{-6}$). For all supraorbital landmarks length and sex influenced shape change (ANCOVA length $F_{1,95} = 22.85$ pvalue = $6.37e^{-6}$; sex $F_{1,95} = 89.34$ p value = $2.45e^{-15}$) and the interaction between length and sex influenced shape change (ANCOVA length:sex $F_{1,94} = 12.36$ p value =0.00067). For eye landmarks, length and sex influenced shape change (ANCOVA sex $F_{1,95} = 89.34$, p value = $2.45e^{-15}$; length $F_{1,95} = 9.33$ p value =0.003) and the interaction between length and sex influenced shape change (ANCOVA length:sex $F_{1,94} = 17.78$ p value = $5.69e^{-5}$).

I also observed and documented the species' first-ever captive instances of fertilization depositing between 96,000 – 134,000 eggs. These eggs were characterized by an adhesive chorion that stuck eggs to each other. Characterization of embryology, hatching, and larval development were documented. Another important finding related the reproductive behavior was the discovery of the males' role in guarding eggs. For the first time, we characterized crucial embryological landmarks. Other major milestones included size at hatching (7.43 mm), and time at first feeding (1 DPH). Endogenous resource utilization was also described by tracking the consumption of yolk and oil globule during embryonic development. More research is needed to optimize hatchery techniques and larviculture, which would significantly contribute to the development of monkeyface pricklebacks as a viable aquaculture species. This study not only enhances our understanding of the species' reproductive behavior but also offers new research avenues crucial for successful captive breeding programs and sustainable aquaculture development.

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INTRODUCTION

Food production can negatively impact the environment in a myriad of ways, from deforestation for terrestrial animal agriculture to overfishing wild fish stocks. With a growing population that is predicted to reach 9.7 billion by 2050, the task of feeding the world, while meeting the need to limit the impacts on the environment, requires urgent and radical transformations of our food systems (Nations, 2015). To meet the projected increasing needs of humanity, food production needs to increase by 25-75% by 2050, with animal agriculture expected to contribute a disproportionate amount of future food via intensification (Hunter et al., 2017). However, based on the recent history of conventional food production methods, this will likely come with significant environmental impacts, such as loss of biodiversity from deforestation and water shortages associated with arable land conversion (Benton et al., 2018).

Nutritional quality from these sources should also be taken into consideration. Recently, terrestrial-raised protein sources have diminished in nutritional value in response to intensive farming practices that seek to maximize production at the expense of other qualities. On the other hand, nutritional quality from aquatically sourced food can provide many human health benefits as they contain micronutrients and essential fatty acids (Tacon and Metian, 2013). In 2017, the consumption of aquatic proteins fed over 3 billion people worldwide and this source is projected to rise in the coming years (FAO, 2022).

According to the Food and Agriculture Organization, we have likely reached the maximum amount of wild seafood harvest that the ocean can sustain (FAO, 2018); landings plateaued in the early 1990s, reaching a maximum of 84.4 million tones. In contrast, aquaculture has increased seafood production globally, with finfish production alone nearly doubling in that same time period (FAO, 2018). This has led to aquaculture becoming the fastest-growing major food production sector in the world, increasing by 8% annually since the 1980s (FAO, 2018), compared to the pork (2.5%) and poultry (5%)

industries (Little et al., 2016). By 2030, it is projected that 62% of all seafood consumed globally will be sourced from aquaculture (Kobayashi et al., 2015).

Aquaculture Trends in the U.S.

Although aquaculture has increased in production, in the last several decades the U.S. aquaculture sector has contracted (-1%), mainly due to increased global competition (Garlock et al., 2020). Low domestic production coupled with high national demand has resulted in the U.S. being the largest net importer of seafood globally in terms of value, sourcing 91% of seafood by volume from overseas (Shamshak et al., 2019). This means that the U.S. is in a seafood production deficit. In an effort to reduce this trade deficit and increase domestic food security and increase resiliency of the sector, the U.S federal government has invested in aquaculture at national and state levels.

California follows the same national trend of importing seafood, which is subject to long, complex, and globalized supply chains that are particularly vulnerable to disruptions by trade disputes. A way to minimize complexity in supply chains and increase seafood resilience in California is by increasing aquaculture activities in the state. This could be beneficial as California imports the most seafood of any other state in the United States (25%), and has the highest consumption per capita in the U.S., making it the largest market for seafood in the U.S. (Love et al., 2021; Fong et al., 2022). However, a vital component of increasing food security via aquaculture involves aquaculture research, which is necessary to inform and aid the progression of the nascent industry in California and nationally.

Aquaculture Sustainability

Overall, the U.S. spent $69.7 billion on seafood in 2017 (Love et al., 2020). Ideally, the U.S. would only import sustainable seafood, however, this is not the case with many different species of unsustainable seafood being imported each year. One species of concern is Unagi, or freshwater eel (*Anguilla* spp.). Unagi is widely eaten around the world with a global value ranging from $550 to $850 million per year. The U.S. imports a fraction of that (~$9 million) (Monticini, 2014), with California

being the largest state importer ($2.5 million of unagi in 2020) ("HS Code 030326 - Trade Statistics, Tariff Rates for Eels (*Anguilla* spp.)," 2022). This fishery is not currently considered a sustainable seafood source (Arai, 2022, 2014; Rahmi et al., 2021). The wild populations that supply the Unagi market have declined dramatically, decreasing by 90-99% of their historical abundance (Lee et al., 2003). Many factors have contributed to this decline, with overfishing being the main driver, but also the complex life cycle of the Anguillidae species, which renders their populations particularly vulnerable to changing environmental conditions.

The eel farming industry has yet to "close the lifecycle" on a commercial scale, meaning that even though they have created freshwater eel hatcheries in Korea and Maine, these hatcheries have had only limited success breeding from successive captive progenies due to the complexity of their larval phase (Shiraishi and Crook, 2015). As a result of the limited research scale successes for captive breeding, most farms have experienced steep production declines due to the aforementioned wild fishery declines.

Monkeyface Prickleback as an Alternative to Freshwater Eel

Many aspects of Anguillid biology and production are challenging for sustainable aquaculture systems, making it prudent to consider an alternative species that can provide a similar seafood product that can be produced in a more sustainable way. Emerging alternative species for aquaculture include organisms that have biological and economic traits that are more suitable for culture, and have or are being tested by practitioners in a culture setting (Wheaton, 2008). Monkeyface pricklebacks (*Cebidichthys violaceus*) are a promising emerging species for aquaculture development because of several innate characteristics. These include herbivory, sedentary behavior, high tolerance to environmental extremes, high fillet-to-carcass ratios, and similarity in product to the existing and high-value unagi (Ralston and Horn, 1986; Horn et al., 1986, 1995; German et al., 2004; German and Horn, 2006; Love, 2011). Monkeyface prickleback (or "Monkeyface eels") are elongated eel-like fishes, in the family Stichaeidae, that live in rocky intertidal and shallow subtidal habitats from Oregon to northern

Baja California (Love, 2011). The species have been reported to live to 18 years in the wild and can attain sizes of 30 inches (76 cm) and weights up to (2.3 kg) (Love, 2011). Monkeyface pricklebacks were frequently fished by indigenous Americans, as they did not need to venture far from shore to find them, and this species is one of the largest resident intertidal fish (Allen and Horn, 2006), scientifically described by Charles Frederic Girard in 1854. Today, a small single commercial fishery supplies restaurants in the San Francisco area. Because commercial landings of this species are so low (100kg - 225kg per year; "Final California Commercial Landings," n.d.), monkeyface pricklebacks have been untapped in the seafood industry to date. Part of the explanation for low catch rates is that commercial harvest is extremely labor intensive as each fish has to be captured by hand, at low tide, using poke pole fishing techniques. This fact, coupled with the biologically intrinsic characteristics of the species, makes monkeyface pricklebacks worthy of investigation as an emerging alternative species for aquaculture.

Monkeyface Pricklebacks Characteristics That are Beneficial for Aquaculture: Herbivory

Feed ingredients and their source is a major economic and environmental consideration in the finfish aquaculture industry, specifically fish meal and fish oil. There has been significant research and environmental advocacy efforts to significantly reduce the use of these ingredients in formulated diets (Oliva-Teles et al., 2015; Shamshak et al., 2019). Currently, the use of fish meal and fish oil for aquaculture consumes 25-35% of wild-caught fish, with demand for these products predicted to increase as aquaculture production expands (FAO, 2020). However, the species used for fish meal and fish oil (typically forage fish species such as anchovies, sardines, and herring) are susceptible to overharvesting and cannot sustainably meet the future demands of the industry (Tacon and Metian, 2009). As a result, the costs of feed ingredients of fish meal and fish oil are predicted to increase as demand outstrips supply. The increased harvest strain on wild fish from the aquaculture industry is creating economic and environmental pressures to use less fish meal and fish oil in formulated diets, while seeking alternative and more sustainable solutions (Schalekamp et al., 2016).

Terrestrial-sourced proteins have been studied as alternative sources for carnivorous fish species in aquaculture for over 40 years, with variable success (Tacon, 1995). Soybean extract is now one of the most economical animal feeds on the market, making up 70% of the feed used in the animal food sector (Lim et al., 2008). However, for aquaculture, a major concern with feeding carnivorous fish plant-based feeds is reduced digestibility that can be attributed to alterations of gut microbiota. Disturbance of the microbial structure and function has been associated with inflammatory intestinal disorders, with gut enteritis serving as a significant cause of mortality in fishes, costing the aquaculture industry more than $1 billion per year (Bakke-McKellep, 2000; Krogdahl et al., 2003). Because monkeyface pricklebacks have already evolved as marine herbivores, their gut microbiome is exceptional at digesting and assimilating algal matter (Horn et al., 1982, 1985, 1995; German et al., 2004; German and Horn, 2006), making them are an excellent candidate as a species that can be feed algal- or plant-based diets.

Monkeyface prickleback juveniles are carnivorous and consume invertebrates. However, once the fish reach a standard length of 4-8 cm (1 year), they transition to a completely herbivorous diet (Montgomery, 1977). Digestive enzyme studies show that this dietary shift to herbivory is genetically predetermined. As the fish increase in size, and are fed a high protein diet, digestive enzymes like carbohydrases (used to digest plant matter) remain at concentrations that are indicative of herbivory (high levels), while digestive enzymes like proteases (used to digest proteins) remain at low levels, even while being fed a high protein diet (German et al., 2004).

Feeding selectivity analysis from field samples of gut contents indicates that monkeyface pricklebacks select annual green (Chlorophyta) and red (Rhodophyta) algal species over brown algae (Phaeophytes) (Horn et al., 1982) and exhibit a more selective diet in the summer months, with a more general diet in winter when resources are scarce (Horn et al., 1982; Miller and Marshall, 1987). In central California, their main diet consists of 14 species of green and red seaweeds (e.g., *Smithora, Microcladia, Porphyra, Ulva, Mazzaella,* etc.; Horn et al., 1982). Laboratory feeding selectivity analysis indicates that their diet preference changes in captivity, whereby the fish prefer red algae over green (Horn et al., 1982).

Captivity Requirements

Other characteristics that make monkeyface pricklebacks desirable for aquaculture include their sedentary nature and tolerance to a wide range of environmental conditions (Ralston and Horn, 1986; Love, 2011). Fish that are sedentary in behavior typically have a lower standard metabolic rate and lower feed consumption, which requires less inputs from culturists compared to fishes that swim and reside in the water column (Botsford and Gossard, 1978). In the wild, monkeyface pricklebacks have a small home range and small space requirements (living in crevices) and can be found in groups under rock outcrops (Helm, 1992). Telemetry tracking data suggests that monkeyface pricklebacks are highly restricted in their movements; they were active less than 1% of the time (~5 minutes a day during a flood tide) and traversed a total area of 2 m^2 (Ralston and Horn, 1986). This characteristic may be beneficial when considering space requirements, stocking density, and energy use, all of which may contribute to an efficient feed conversion ratio (FCR) of this species. The FCR of monkeyface pricklebacks was evaluated by Horn et al. (1995), revealing that monkeyface pricklebacks have an FCR of 1.36 when fed a natural diet of seaweed. However, when provided with a formulated diet supplemented with a modest amount of protein, they exhibited faster growth and achieved an improved FCR of 0.96 (Horn et al., 1995).

Stress Tolerance

Having a fish tolerant to a range of water temperatures and dissolved oxygen levels is useful in aquaculture due to the high densities that are often typical of the industry. Monkeyface pricklebacks survive well in waters of varying temperatures (ranging from 9-25ºC) and can respire aerially for up to 18-35 hours when held out of the water (Horn and Riegle, 1981; Love, 2011). These characteristics are all beneficial for aquaculture because changes in water conditions are less likely to impact survivability as acutely as other farmed species.

Monkeyface Reproduction and Early Life History

Despite the relatively abundant research on monkeyface prickleback digestive capabilities in the wild, limited research has been conducted on their reproductive capabilities in a captive setting. While there is some basic understanding of monkeyface prickleback reproduction based on ecological studies (Miller and Marshall, 1987; Marshall and Echeverria, 1992), this information is largely inadequate to reliably predict and induce spawning of the species. Nonetheless, this information is useful to design captive spawning experiments to understand the necessary elements. Monkeyface pricklebacks are oviparous and reproduce by laying small demersal eggs that are deposited on rocky surfaces to which they adhere (Miller and Marshall, 1987). Spawning for monkeyface pricklebacks is reported to occur from January to August, with peak spawning observed from February to April, and with older fish spawning earlier in the season (Marshall and Echeverria, 1992). Females have been observed to contain between 17,000 and 46,000 eggs, reaching first sexual maturity in four years (36 cm standard length). Populations reach 50% maturity at 5 years (39 cm) and 100% maturity at 7 years (45cm) (Marshall and Echeverria, 1992).

Parental care has been observed in the species, however there has not been sufficient examination of reproductive behavior to clearly identify which sex guards the egg mass (Lea and Reilly, 2001). Observations have been reported wherein one or both parents are observed coiled around an egg mass (Fitch et al., 1971). Close relatives of the monkeyface prickleback, the black prickleback (*Xiphister atropurpureus*) and rock prickleback (*Xiphister mucosus*) exhibit male parental care and can tend up to three egg masses from different females at one time (Marliave and DeMartini, 1977). Observations of guarding in captivity can shed light on this reproductive behavior.

Nesting and parental care are common, widespread, and diverse in fish (Sargent and Gross, 1986; Hudson, 1998). This behavior is considered advantageous for species with demersal and/or adhesive eggs, which are easily predated upon by benthic organisms such as sea stars and fish. In 21% of bony fish families, parental care extends beyond fertilization and has evolved in many ways to increase the survival of offspring. It is a common strategy for monogamous mating strategies, as well as territorial fishes

(Gross and Sargent, 1985). There are direct advantages to offspring as well, like parental egg fanning using the elongated body and caudal fin. This fanning behavior provides water motion and oxygenation of the eggs, enhancing the health of the developing embryos, and also dislodging and removing dead eggs by the attending male or female. The attending parent will also defend the nest from any potential predators (Hudson, 1998); however, there are disadvantages to parental care in aquaculture because of the potentially complex courtship phase, as seen in the demersal spawning wolf eel (Marliave, 1987). Courtship for spawning may require certain substrates or other environmental cues, or it may require desirable traits seen in the male or female, all of which, may be difficult factors to identify in captivity.

There are many other knowledge gaps beyond spawning. Embryological development has not been investigated for the species and therefore, egg development and the timing and requirements for successful incubation until hatching for monkeyface pricklebacks are unknown, as are the length of the larval period and nutritional requirements of larvae after hatching. Other knowledge of the early life history for this species indicates that larvae settle out of the planktonic phase back into intertidal locations at sizes around 1.7 cm to 2.2 cm in length (Miller and Marshall, 1987).

Research Motivation

Controlling reproduction is critical for new species for aquaculture, therefore research on spawning and early life history are necessary for developing hatchery methods. Many knowledge gaps surround monkeyface prickleback reproduction and early life history. This research aims to fill many knowledge gaps needed for developing culture techniques for the species. By investigating the objectives below, this study attempts to understand monkeyface prickleback reproduction and to test whether monkeyface pricklebacks are a viable aquaculture species.

OBJECTIVES

This study investigates different aspects of culture for monkeyface prickleback (*Cebidichthys violaceous*).

Objective 1. To determine if sexual dimorphism through external morphological features can be used to reliably identify male and female monkeyface pricklebacks, which may facilitate broodstock management in a captive environment. This objective is investigated in Chapter 1.

Objective 2. To determine optimal conditions for spawning, elucidate parental care functions during egg incubation, create an embryological development timeline, and characterize larval development. This objective is investigated in Chapter 2.

Chapter 1. Identification of Sexual Dimorphism in Monkeyface Pricklebacks

RATIONALE AND BACKGROUND

One of the major impediments to developing a new aquaculture species is the reliable production and supply of juveniles (Silva et al., 2008). While there are many aspects involved in the production of juveniles in aquaculture, the process usually begins with broodstock and their management. In particular, new candidate species for aquaculture historically use natural spawning techniques, already ripe and running fish from the wild, or fish that generally spawn continuously (Harvey and Hoar, 1979; Pillay, 1990; Landau, 1992). To generate fry, most wild and captive broodstock managers must provide adequate conditions for reproductive strategies that often include creating or inducing a favorable environment and broodstock sex ratios (Cnaani and Sivan, 2009).

In order to determine appropriate sex ratios, a reliable and objective sex identification method is needed. In species that do not have obvious sexual dimorphism, commonly employed methods of sex identification, such as ultrasonography and canulation, have been used on many different species of fish, but these techniques have disadvantages (Martin et al., 1983; Ross, 1984; Karlsen and Holm, 1994; Martin-Robichaud and Rommens, 2001). While ultrasonography can be quite effective at determining the presence of an ovary, this method may not be viable during resting phases in reproductive cycles where ovaries and testis are greatly reduced in size and harder to detect with ultrasound. Additionally, the equipment and training necessary for fish sonography can be costly and impractical for the culturist (Slembrouck et al., 2019). Canulation methods for sex identification are a common method for many species, that involves inserting a small bore flexible tube inside the fish's gonopore to retrieve gonad tissue for examination (Axelsson and Fritsche, 1994). This invasive disturbance can be detrimental to broodstock health and conditioning, imparting unwanted stress on the fish (Davison et al., 2023). Being able to distinctly identify sex using other more discrete visual characteristics, such as head shape and

other external morphological characters is a potential effective solution for helping fish farmers accurately identify sex and determine broodstock sex ratios.

While the monkeyface prickleback is commonly found in public aquariums, there has been no previous scientific interest in breeding this species for aquaculture. The lack of reproductive knowledge for the species has created a large knowledge gap for information related to sex identification and very little is known about reproduction of the species, making monkeyface pricklebacks in need of methods for sexual identification. In regards to sexual identification, fortunately for some species of fish, evolutionary forces of sexual selection and specifically, intersexual selection (mate choice, usually by females), have led to adaptations of secondary sexual characteristics between males and females (Shine, 1990; Berns, 2013). Examples of these adaptations may consist of sexual ornamentations, behavioral displays between sexes, or other morphologically based sexual dimorphisms. Any one of these sexual dimorphisms are helpful to culturists, or other scientists, because visual identification is the fastest and simplest way to identify the sex of an individual.

Fish possess many sexual determination strategies, which fall into three main categories: color pattern, size, and shape differences. Color pattern differentiation is a prevalent form of sexual dimorphism in fish, particularly in species like wrasses, which exhibit both size and color-based sexual dimorphism (Roede, 1972). This phenomenon is also observed in fish species where males develop distinct breeding colors, such as the painted greenling *Oxylebius pictus* (Edward, 1986) and Nassau grouper *Epinephelus striatus* (Archer et al., 2012). The kelp greenling (*Hexagrammos decagrammus*) provides a local example with distinct male and female color patterns (Edward, 1986). However, monkeyface pricklebacks are considered monomorphic and defy color-based sexual dimorphism, necessitating alternative methods of sexual identification.

Other strategies such as size and shape variation among males and females may be diagnostic for sex determination in fish species that are monomorphic in their color patters. For example, body size differences (sexual size dimorphism) and shape (morphologically based sexual dimorphism) differences occur between the sexes in some fish species (Parker, 1992). Size-based sexual dimorphism relies on the

relationship between length and sex, where either males or females are larger than the opposite sex. Accurate sex determination is only likely, however, when size extremes are present, where males or females are much larger than the other sex (Parker, 1992). Shape-based sexual differences can be more useful for sex determination across similarly sized fish. These types of sexual dimorphisms have been used in various different studies and applications, and are helpful for broodstock management or population studies looking at demographics of a species (Herler et al., 2010; Martínez-Chávez et al., 2018). Marshal and Echeverria (1992) documented a size-based sexual dimorphism in wild monkeyface pricklebacks, reporting that males are slightly larger than females. Beyond the aforementioned size-based difference, monkeyface pricklebacks are generally considered to be sexually monomorphic due in large part to a lack of detailed study. The size difference between male and female monkeyface pricklebacks is less extreme, and there is a large amount of overlap in size for the two sexes. When this is the case, size-based sexual dimorphism becomes less accurate. Having a more distinct sexual marker is helpful, and two examples of distinct morphological sexual dimorphisms have been seen in two species of fish that are in the same phylogenetic lineage as the monkeyface prickleback. The closely related high cockscomb *Anoplarchus purpurescens* males have a larger head and more prominent comb (forehead feature across the nape) that helps visually distinguish sex within the species (Coleman, 1992). Another more distant phylogenetic relative in the Scorpaeniformes order is the wolf eel *Anarrhichthys oceallatus*. Although this relative is more distantly related phylogenetically, wolf eels closely share other characteristics with monkeyface pricklebacks like their habitat preference (benthic dwelling), morphology (elongated fish), as well as a prominent supraorbital crest, which is a sexually dimorphic trait. The wolf eel's supraorbital crest is a shape-based sexually dimorphic feature only occurring during the spawning season on male fish (Marliave, 1987). These related characteristics prompted my study to investigate whether monkeyface pricklebacks share any analogous morphological features in regard to their head shape.

Geometric morphometrics is commonly used to analyze body shape or other morphological characteristics (Begg and Waldman, 1999; Klingenberg, 2011; Zelditch et al., 2012). This technique can be used to verify conspicuous sexual differences or identify cryptic qualitative differences with the power

of statistics (Zelditch et al., 2012). Geometric morphometric methods have been used for many different studies on aquatic species, from population analysis, phenotypic plasticity, phylogenetics, to comparing morphology between geographically isolated populations. (Herler et al., 2010; Hopkins and Thurman, 2010; Silva et al., 2010; Martínez-Chávez et al., 2018). This method has also been used to identify phenotypic variation within a species (O'Reilly and Horn, 2004; Lawing and Polly, 2010), as well document and statistically verify sexual dimorphism in fish and marine invertebrates (Dorado et al., 2012; Accioly et al., 2014). Geometric morphometric methods may increase the power to discriminate between sexes because it can identify minimal morphological differences that go unnoticed by traditional observational or morphometric analysis. These techniques are helpful for culturists and have led to many sexual dimorphism studies that aided sex identification in aquaculture species. This includes species that are well developed in the aquaculture industry, such as white shrimp, sturgeon, and tilapia (Accioly et al., 2014; Oponda et al., 2017; Balazadeh and Litvak, 2018), as well as lesser known or up and coming cultured species such as the giant gourami (Slembrouck et al., 2019) and cobia (Molina et al., 2018).

While monkeyface pricklebacks are generally found to be monomorphic, males and females both possess a supraorbital crest similar to the sexually dimorphic crest found in the wolf eel. This chapter uses geometric morphometrics to investigate geometric differences in head shapes of monkeyface pricklebacks (specifically the supraorbital crest), and whether these differences can be used as discrete sexually dimorphic markers. This investigation aims to aid future culturists in sexual identification of broodstock and ultimately to support the development of hatchery protocols for the species. Specifically, this study used geometric morphometrics to answer 3 questions: (1) Does the head shape differ between male and female monkeyface pricklebacks? (2) Is the supraorbital crest a sexually dimorphic feature for monkeyface pricklebacks? (3) What is the accuracy of ultrasound techniques compared to geometric morphometric techniques for identifying sex?

METHODS

Fish Collection and Transportation

For this study, morphometric information was acquired from 100 monkeyface pricklebacks. Fish collections occurred between September 2020 through February 2021 at multiple locations along the central California coast during low tide. Fish were collected utilizing poke-pole fishing techniques, which involved prodding a baited pole into caves where monkeyface pricklebacks reside in the intertidal, focusing on sites where they are known to be abundant at low tide. These sites included Half Moon Bay, Carmel, Moss Landing, and San Simeon, CA (**Figure 1**). Collections occurred bi-weekly when the low tide was near 0.15 m or below the mean low tide. Poke poles used for the collection were made up of 182 cm long x 1.27 cm diameter PVC, wire hanger, and monofilament leader line with baited Gomakatsu size 2 barbless octopus hooks. The primary bait used was 3 cm pieces of fresh or frozen squid. Juvenile monkeyface collected from San Simeon, CA, were collected using dip nets at low tides of (0.-5) or lower than the mean low tide mark. 19 juveniles were included in the morphometric study to observe how head shape changes with the size of the fish.

Broodstock were transported to Moss Landing Marine Labs (MLML) in aerated containers and placed in holding tanks to recover. Upon arrival, each fish was visually assessed for health and monitored. Until the desired sample size was collected, fish were held and cared for in three 3,400 L holding tanks stocked up to n=50 per tank. Tanks were equipped with cinder blocks and PVC habitats simulating natural cave structures, fish were fed various species of algae that make up their natural diet every other day, and tanks were cleaned weekly.

Sex Identification from Ultrasound

At the beginning of the study, the sex of fish was identified using ultrasound techniques. To complete a successful sonogram, fish were anesthetized in a bath solution of 50 mg/L of MS-222 (Tricaine methane sulfonate) in seawater until they did not respond to grasping of the caudal fin. While

sedated, fish were placed on their right side, and a portable ultrasound machine (GE Venue 40 portable ultrasound machine with GE L8-18i transducer probe) was used around the left abdominal region to locate the presence/absence of an ovary. **Figure 2** displays a sample image of one ovary being located using ultrasound equipment. Sexual identification using this technique was based on observations of an ovary (females), while males were determined from the absence of any visible reproductive organ. After sex identification using the ultrasound, fish were measured, weighed, and Passive Integrated Transponder (PIT) tagged using Biomark (APT12 FDX-B) 12 mm internal tags. The accuracy of this method for sex determination was tested by comparing sex identification from ultrasound observations to the confirmed sex following biological dissections at the completion of the study. The accuracy of this method of sex identification was then compared to geometric morphometric sex identification methods, as well as blind visual determinations of sex using the photographs.

Photographic Methods for Morphometrics

This study implemented geometric morphometrics techniques to analyze the head shape, including the supraorbital crest, for sexual dimorphism. Morphometric analysis is a tool for quantifying and analyzing shape differences (Zelditch et al., 2012). Shape is mathematically defined as the geometrical information of an object that remains when location, scale, and rotational effects are filtered (Dryden and Mardia, 2016). Geometric morphometrics uses shape coordinates of a particular body part (anatomical landmark) that are digitized from a photograph in which they can be analyzed in multidimensional space, to test whether significant variation exists between species or sexes.

The 100 specimens used for geometric morphometrics consisted of a combination of sexually mature fish (n=81) and sub-adults (n=19). The determination of sexual maturity was based on the length at sexual maturity relationship described by (Marshall and Echeverria, 1992), where size at first maturity is 36 cm, size at 50% maturity is 39 cm, and size at 100% maturity is 45 cm. For this morphometric study, fish were anesthetized in a bath solution of 50 mg/L of MS-222 (Tricaine methane sulfonate) in seawater until they did not respond to grasping of the caudal fin. While sedated, fish were supported on a V-shaped

cradle resting above a length board and photographs were taken to ensure the accuracy of landmark location during digitization. A lateral image of the left side of the sedated fish was taken. The fish were then placed in a recovery tank and then returned to their holding tanks. Photographs then underwent processing and placement of digital landmarks.

Landmarks in geometric morphometrics are points that correspond to a specific area in the anatomy of a structure (Rohlf, 2002). Landmarks are ultimately used to extract the geometric coordinates from the digital photographs for comparative analysis. Photographs were first formatted to a TPS file using the software tpsutil64, then cranial features were digitally landmarked using tpsDig232 software. Scale and landmarks were set and placed on each photo. **Figure 3** displays an example of areas landmarked, consisting of 25 landmarks, with 15 landmarks along the supraorbital crest, generating a landmark curve for investigation of this potentially sexually dimorphic feature. Having many landmarks on this curve increased the resolution of the shape and ensures that it is accurately captured for analysis. This landmark curve consisted of landmarks 11-25 (blue line denoting the landmark curve on head crest; **Figure 3**). This feature is referred to throughout as the supraorbital crest or head crest. Other cranial landmarks included: a landmark point on the snout (landmark #1), landmarks at the anterior and posterior insertions of the supraorbital crest (#2, 3), insertion of the dorsal fin (landmark #4), operculum (landmark #5), maxilla (landmark #6), and 4 landmarks around the eye (landmarks #7-10). While landmarks 11-25 were chosen to investigate the supraorbital crest as a potentially sexually dimorphic feature, all other landmarks were chosen because they represent distinct points around the head that can be easily replicated during landmarking, they illustrate the general shape of the head, and represent anatomical parts that are commonly investigated in geometric morphometrics. For example, the distance between the tip of the maxilla (#6) and the insertion of the dorsal fin (#4) can reveal how wide or narrow the head shape is. All methods for landmark criteria were based off of the work of (Zelditch et al., 2012).

Statistical Analyses

Question #1: Does the head shape differ between male and female monkeyface pricklebacks? This analysis used and compared all digitized landmarks listed above to test the general difference in head shape between males and females. Question #2: Is the supraorbital crest a sexually dimorphic feature for monkeyface pricklebacks? This analysis isolated the head crest landmarks (landmarks #11-25) and used the same multivariate analyses to test whether the supraorbital crest is shaped differently for males and females, making it a sexually dimorphic feature. Question #3: What is the accuracy of ultrasound techniques compared to geometric morphometric techniques for identifying sex? The accuracy of the ultrasound was determined by comparing sex identification results from the ultrasound to the sex determined upon fish dissection. Ultrasound accuracy and geometric morphometrics accuracy were compared to the accuracy of a visual determination of sex using the same photographs of fish used in the geometric morphometrics, and asking individuals to assess the sex of an individual with the only knowledge that monkeyface pricklebacks had a sexually dimorphic supraorbital crest.

Shape coordinates were calculated from landmarked data provided by tpsDig232 for analyses. All statistical analyses were run using MorphoJ software, a graphical user interface platform that can perform a broad range of morphometric analyses on two or three dimensional landmark data (Klingenberg, 2011). Analysis of morphometrics requires analyzing multivariate data, which was utilized in the MorphoJ software. Geometric morphometric software used for this study is publicly available at http://www.sbmorphometrics.org/.

Before any multivariate analyses were conducted, a Procrustes superimposition was performed on both groups of landmarks. Procrustes superimposition centers each landmark to its centroid, removing any location factors. Absence of location allows the Procrustes superimposition to then scale all landmarks around the same centroid and rotation scale factor generating Procrustes coordinates (**Figure 3**). A covariance matrix composed of the Procrustes coordinates was then used to run multivariate analyses. These first two steps were crucial in preparing the landmarking data for multivariate and ordination comparison and analysis.

A Procrustes ANOVA is a type of multivariate ANOVA that quantifies amounts of shape variation in a factor of interest. Because this study was interested in how shape varies between males and females, a Procrustes's ANOVA was performed with sex as a categorical classifier. Shape variation was compared across all landmarks, as well as isolating only the supraorbital crest landmarks to evaluate if the supraorbital crest is a discrete sexually dimorphic feature. Other unexpected shape changes that were observed, such as the arrangement of the eye in relation to its distance from the fish's nape was investigated further using the same statistical methods outlined.

A Canonical Variate Analysis (CVA) was used to test how shape arrangements change between males and females. A CVA is commonly used in sexual dimorphism studies utilizing geometric morphometric techniques because it provides a type of multivariate comparison analysis that maximizes the separation of specified groups (sex) (Zelditch et al., 2012). The CVA also generates a mahalanobis distance (D^2) with a parametric p-value (10000 permutations). The mahalanobis distance describes the distance between a point and a distribution, which is understood as the scale of shape variation (i.e., what is the magnitude of shape change). In other terms, the mahalanobis distance is the multivariate equivalent to the z-score produced from 2 dimensional statistics that informs the significance and magnitude of variation (Zelditch et al., 2012). The CVA also generates a Procrustes distance between groups, with a parametric p-value that explains the observed shape distance between Procrustes coordinates. This analysis provides several different graphical outputs from the CVA that help visualize where the maximized shape changes occurred between groups. To analyze how the CVA scores from this analysis changes as a function of length, an ANCOVA was performed investigating the interaction that length and sex have on CV1 scores, as well as the combined interaction length and sex can have on the CV1 scores. Tukey Post Hoc tests further revealed how these covariates influence shape change (CV1 scores). The ANCOVA and Post Hoc tests were performed in RStudio.

To understand how accurate geometric morphometrics are for sex determination in monkeyface pricklebacks, a Discriminant Function Analysis (DFA) was performed. A DFA with cross-validation computes the accuracy of group placement for sexual identification using geometric morphometrics

(Peter, 1967). A DFA is similar to a CVA in that it examines the separation between two groups of observations (male-female). The difference is that a DFA also implements "leave-one-out cross-validation", which explains the reliably of using geometric morphometrics for sex identification given the various landmarks used in the analysis (Peter, 1967). By comparing the accuracy of ultrasound to that of geometric morphometric techniques and visual identification, I aimed provide valuable insights to culturists regarding the most suitable techniques for their sexual identification needs.

RESULTS

Shape Variation Between Sexes

When considering all the digitized landmarks in this study, the results suggest that male and female head shapes are significantly different (Procrustes ANOVA, $F_{46,4508} = 25.76$, p-value < 0.0001; **Table 1**). Running the same test on isolated supraorbital crest landmarks also detected significant differences between males and females (Procrustes ANOVA, $F_{30,2940} = 5.96$, p-value < 0.001; **Table 1)**. The Procrustes ANOVA also suggests that eye placement differs for females and males (Procrustes ANOVA, $F_{8,392} = 49.04$, p-value < 0.0001) These results indicate that head shape, the shape of the supraorbital crest, and eye placement are different between the two sexes.

Magnitude of Shape Difference and Variation of Landmark Arrangement Between the Sexes

When using sex as a classifier, the CVA detected significant landmark separation between males and females for all digitized landmarks (CVA, $D^2 = 3.46$, p-value < 0.0001; **Table 1**). A similar significant discrimination between male and female fish was observed when using only supraorbital crest landmarks (CVA, $D^2 = 1.95$, p-value < 0.0001; **Table 1**). The CVA also detected variation of eye placement in respect to the nape for male and female (CVA, $D^2 = 2.26$. p-value < 0.0001; **Table 1**). Each CVA generated two groups of Procrustes coordinates (mean weighted placement of landmarks for male and female groups) represented by CV1. These two groups of Procrustes coordinates were visualized

using a wireframe graph that links the anatomical configurations of landmarks to help illustrate each different shape variation seen from CVA analysis. Looking at the wireframe graphs, one can see there were many shape change differences between male and female fish, where males (blue) have an overall different head shape with a much larger supraorbital crest (landmarks 11-25), and smaller sunken in eye shape (landmarks 7-10) relative to the nape of the fish compared to females (**Figure 4**). Next to each CVA wireframe configuration there is a corresponding frequency of variation histogram displaying the differences in variation between groups. This histogram displays the frequency of separation between males and females using the canonical variate score (CV1) calculated from the CVA. Dissimilar CV1 scores represent the computed shape difference between males and female fish. All histograms for each CVA show a significant degree of separation between males and females (**Figure 4).**

Shape Change as a Function of Length

Scatter plots of computed regression points were plotted for all resulting CV1 scores from each CVA to as CV1 scores visualize how canonical variate 1 (male and female shape difference) is related to fish total length **(Figure 5)**. Three similar trends resulted from all plots that display differences in male and female anatomical shapes (CV1 scores) for all analyses (all landmarks, supraorbital crest landmarks, and eye landmarks) The three plots collectively demonstrate a correlation between shape difference, length, and sex, as well as shape change measured by the CV1 score. This correlation becomes evident when considering the size of the fish: while the fish are small in size, both males and females exhibit similar CV1 scores. However, as the fish grow, a noticeable pattern emerges. In males, there is a positive correlation between CV1 shape and fish length, indicating that as males increase in size, their shape tends to change in a more positive direction. On the other hand, females exhibit a distinct pattern of shape change. For females, the CV1 scores demonstrate opposing trends, becoming more negative as fish length increases. Ultimately, these observations suggest a relationship between fish length and the presence of sexual dimorphism .NCOVA for all three characteristics support this size shape relationship. For all

landmarks sex influenced shape change (ANCOVA, sex: $F_{1,95}$ =344.53, p value <$2.2e^{-16}$) and the interaction between length and sex influenced shape change (ANCOVA length:sex $F_{1,94}$=24.26 p value <$3.59e^{-6}$). For all supraorbital landmarks length and sex influenced shape change (ANCOVA length $F_{1,95}$ = 22.85 p value = $6.37e^{-6}$; sex $F_{1,95}$ = 89.34 p value =$2.45e^{-15}$) and the interaction between length and sex influenced shape change (ANCOVA length:sex $F_{1,94}$ = 12.36 p value =0.00067). For eye landmarks, length and sex influenced shape change (ANCOVA sex $F_{1,95}$ = 89.34, p value = $2.45e^{-15}$; length $F_{1,95}$ = 9.33 p value =0.003) and the interaction between length and sex influenced shape change (ANCOVA length:sex $F_{1,94}$ = 17.78 p value = $5.69e^{-5}$).

Linear regression equations for the relationship between length and shape difference depicted in **Figure 5** for all characteristics. The differing slopes in each regression indicate the shape vs. length relationship for males and females are different which supports the idea of sexual dimorphism. For all landmarks the slopes increase with length while in females the slope decreases with length. For the supraorbital crest the CV1 slope increases for males with length while it decreases with length for females. For eye landmarks CV1 slope decreases for males with length with it increases with length for females.

Accuracy of Sex Identification

The DFA resulted in a 73.9% accuracy when using all landmarks for sex identification of males and females. However, this geometric morphometrics accuracy increases with fish length as larger fish exhibit larger shape differences between the sexes seen by divergent CV1 scores for males and females as size increases (**Figure 5**). The DFA showed that sexual identification becomes 78.6% accurate when comparing fish ≥ 36 cm in length. The DFA accuracy increased even further to 82.65% and 81.45% when grouping fish ≥ 39 cm ≥ 45 cm, respectively (**Figure 6**). The accuracy detected when using the same cross-validation method for only supraorbital crest landmarks was found to be slightly weaker at 64.9% for all fish, and 68.65 %, 69.2%, and 70% for fish ≥ 36 cm, 39 cm, and 45 cm, respectively (**Figure 6**).

The ultrasound method for evaluating sex of fish proved to be a viable technique for determining the sex of monkeyface pricklebacks. To ground truth sex identifications made with the ultrasound method, all experimental fish we dissected at the end of study to observe the presence of ovary or testes tissue. Of the 79 fish initially examined with sonography only 3 individuals were incorrectly assigned to male or female based on sonography (96.2% accuracy) (**Figure 6**). The accuracy of the visual assessment resulted in 75.3% accuracy for all fish, increasing with fish length to 78.6%, 79.6%, and 81.4% for fish ≥ 36 cm, ≥ 39 cm, and ≥ 45 cm respectively (**Figure 6)**.

DISCUSSION

The monkeyface prickleback is a new aquaculture species and has never been bred in captivity. The lack of research into the species' reproductive capacity creates knowledge gaps that are needed for captive breeding programs. One main requirement within a captive breeding program is sexual identification of males and females. The results of this study suggest that an existing sexual dimorphism in cranial features can be used for sexual identification. While the results indicate males and females are better differentiated when using all cranial landmarks, morphological features are most effective for sex discrimination if unique characteristics can be used in diagnostic keys or guides. The supraorbital crest in monkeyface pricklebacks was found to be a robust morphological discriminator between the sexes in this study. After conducting geometric morphometric analysis, this study identified at least two sexually dimorphic characteristics: the supraorbital crest, and the placement of the eye relative to the nape.

Several other studies have investigated the sexual dimorphism of the head shape or supraorbital crest in fish. The shape and size of this crest can be influenced by a diversity of factors, including sexual selection, parental effort, and hormone levels due to reproduction or maturation. Marliave (1987) describe the morphology of the supraorbital crest in wolf eel males,

finding that during the breeding season males had a larger and more prominent supraorbital crest than females. The researcher suggested that this sexual dimorphism may be related to the sexual selection for males with larger and more elaborate crests, as a signal of their fitness to potential mates. The California sheephead, *Semicossyphus pulcher*, also showcases strong sexual dimorphism, with males being larger and more vibrant than females with a large, crested head. This sexual dimorphism can be attributed to sexual selection and is likely driven by territorial behavior and group dominance hierarchies, because large males compete for territorial space and access to mate with groups of females (Adreani et al., 2004).

While sexual dimorphism of the supraorbital crest is a common phenomenon in fish – with males often having larger and more pronounced crests than females – the size and shape of the crest may be linked to certain qualities associated with reproduction and male parental care in fish. One study specifically compared sexually dimorphic traits such as head crests in male *Salaria pavo* that exhibit parental care for eggs. The study reported that the size of the supraorbital crest expressed appeared to be positively related to sperm number (Pizzolon et al., 2012). However, the authors also found that the size of supraorbital crest in these males is negatively related with the amount of parental care effort (Pizzolon et al., 2012), suggesting that there may not be a clear relationship between sexual dimorphism and parental care. Another example involves male cichlids *Cichlasoma citrinellum*, where swelling of the supraorbital crest occurs prior to spawning in response to fluctuations in reproductive hormone levels (Bleick, 1975). The authors reported they could induce supraorbital crest growth with mammalian gonadotropins (HCG or ovine LH). This study suggests that in some cases supraorbital crest morphology can be largely related to reproduction. Lastly, a study by Kajiura et al. (2005) investigated the sexual dimorphism of the supraorbital crest in bonnethead sharks *Sphyrna*

tiburo. They reported that males had larger and more pronounced supraorbital crests than females, and the size and onset of the crest was also influenced by the onset of clasper development and maturation. This list of examples illustrates that sexual dimorphism of the supraorbital crest can be widespread and reasons for dimorphism can be diverse. It is also important to note that many of the factors that may contribute to sexual dimorphisms are not necessarily mutually exclusive and many could be contributing to the observed differences between males and female fish.

Considering these intriguing findings, one cannot help but draw connections to the world of monkeyface pricklebacks. These fish, known for their distinct facial features, have the remarkable behavior exhibited by their males, who parent and guard and care for egg masses. In light of this unique reproductive strategy, it becomes plausible to speculate that the evolution of larger supraorbital crests in male monkeyface pricklebacks might have arisen through the process of sexual selection. Female monkeyface pricklebacks, in their quest for optimal mates, could have favored larger males displaying larger crests in mature males as a sign of their ability to potentially provide enhanced parental care and protect the precious egg masses from predators. One can explore the other 'hard hitting' potential implications for the monkeyface prickleback's supraorbital crest by comparing it with other fish species, such as the bumphead parrotfish *Bolbometopon muricatum*. In this species, males exhibit high-speed swimming behaviors and engage in intentional collisions, utilizing their supraorbital crest as a protective mechanism (Callaway, 2012). However, different from *Bolbometopon muricatum*, the monkeyface prickleback head crest is made up of softer fatty tissue and is unlikely used for actual defense or fighting but rather a signal of male dominance or maturity to portray its status to both other males and courting females.

The relationship between size and reproductive success in the context of sexual dimorphism is a complex topic that requires further investigation beyond this study. Although the current study shed some light on the matter by demonstrating a sexual dimorphism that is related to size. There is also still much to uncover, because this study was unable to link reproductive success to the size of fish or size of the headcrest. However, this study uncovered this relationship between size and sexual dimorphism can steer future research. One could further explore the potential impact of size-assorted mating, as observed in related species like the high cockscomb *Anaplarchus purpurecens* (Coleman, 1992). One can also look into how the size of supraorbital crest is related to successful spawning. The door to future research in captive spawning and sexual dimorphism of monkeyface pricklebacks is now wide open for future investigations into these relationships.

One unexpected finding in this study was the differences in eye arrangement between the sexes, which adds an intriguing layer to the exploration of sexual dimorphism. It is important to consider that the reliability of these eye landmarks as sexual dimorphisms has been questioned due to landmark placement on spherical characteristics, making them "semi-landmarks" that may not be suitable for geometric morphometric comparison. These semi-landmarks do not represent discrete anatomical loci and do not adhere to the rule of landmark homology (Zelditch et al., 2012). However, they can still provide insights into general shifts or size changes, as demonstrated in previous studies examining the effects of freezing and alcohol preservation techniques on fish eyes (Berbel-Filho et al., 2013). In this study, eye semi-landmarks were used to examine changes in eye placement between the sexes, specifically focusing on landmark configuration #7-10, which fully encircled the eye (**Figure 3)**. While the statistical reliability of these semi-landmark movements may be debatable based on the concept of homology proposed

by Zelditch et al. (2012), the results pertaining to the differences in eye placement remain significant for external evaluations of individuals.

The question arises: Why do monkeyface pricklebacks exhibit sexual dimorphism in eye shape? One possibility is that the differences in eye size and shape are related to behavioral disparities between males and females, such as aggression, courtship, or mate choice. Larger eyes may enhance males' ability to locate and pursue females effectively, while distinctive eye shape could serve as a visual signal of dominance or attractiveness. Alternatively, sexual dimorphism in fish eyes may be influenced by physiological or anatomical disparities between males and females. Hormone levels or variations in skull structure could potentially impact eye size and shape. Furthermore, divergent visual needs or abilities between the sexes, driven by their respective roles in reproduction or feeding, may contribute to the observed sexual dimorphism. Many of these drivers of sexual dimorphism are seen in different examples of fish. For instance, three species of rockfish *Sebastes fluvidus, S. mystinus. and S. serranoides* all exhibit eye sexual dimorphism, where males who tend to be smaller have proportionally larger eyes, which allow them to compete on the same level as females for elusive prey items (Echeverria, 1986). Male flatfish *Bothus robinsi* have been found to have larger space between the two eyes that is thought to help females distinguish males for potential mates (Kobelkowsky, 2004). Given the complex and diverse nature of sexual dimorphism in monkeyface prickleback eyes, it is crucial to note that the specific reasons for eye sexual dimorphisms were not further investigated in this study due to limited reproductive information available for the species. However, future investigations of this dimorphism may help select broodstock males with hopes of increasing successful courtship and reproductive success.

The accuracy of male and female sexual identification using geometric morphometrics was comparable to other studies of sexual dimorphism using a variety of traits. The benefits of visual identification of sexual discriminators also align with other studies that have recently utilized geometric morphometrics suggesting it is an economical and adaptable method for aquaculture purposes. For example, Russian sturgeon grown for caviar, previously characterized as monomorphic, were recently observed to be sexually dimorphic after applying geometric morphometrics to investigate differences in cranial characteristics. Balazadeh et al. (2018) used a CVA to identify significant sexual dimorphisms in which females have a shorter head with a snout closing in further to the eyes, while males had longer but more laterally compressed head. This same study also used a DFA and found it was able to correctly classify 83% of males and females (Balazadeh and Litvak, 2018). The authors attributed shape change to sturgeon mating strategies, where male-male reproductive competition may have led to the narrowing of the head for increased mate access to work through groups of competing males over a single female. This study claims that geometric morphometrics method for sex identification is more accessible to users and is less resource intensive than hormonal sex identification methods in aquaculture production.

Similar results and benefits from investigating sexual shape dimorphism using geometric morphometrics have been reported in juvenile cultured cobia (Molina et al., 2018). The authors used a CVA and DFA to analyze the head and body of juvenile cobia and found that geometric morphometrics uncovered shape differences between juvenile males and females. Males were found to have extended length of dorsal and anal spines, where females have a more vertically stretched and deeper medial portion and larger anal fin than males. The female shape dimorphism was attributed to sexual selection forces on the abdomen for an increase in the

abdominal cavity, where reproductive organs are located, allowing more room for developing gonads when they reach maturity. This trait is selected for because larger gonads result in an increase in fecundity for female cobia once they reach maturity. The cross-validation matrix resulted in 83.33% correct sex classification, similar to this study on monkeyface pricklebacks. In cobia, because females ultimately grow much faster and larger than males, a mono-sex culture is preferred for farmers. The sexual shape dimorphisms were found to help farmers take males out of production early, which increases unit yields by having all fast-growing female stocks. The benefit of this visual early morphometric classification has helped cobia farmers in low infrastructure areas and regions to increase cultivation of this species quickly and cheaply.

The application of geometric morphometrics in the identification and characterization of sexual dimorphism in monkeyface pricklebacks has brought about a significant contribution to the aquaculture industry. As a novel technique, geometric morphometrics has exhibited notable efficacy in discerning the often-subtle sexual dimorphism present in broodstock. This fresh insight into the sexual dimorphism of monkeyface pricklebacks is of immense importance for broodstock selection, especially when assessing larger specimens and other fish above the size at first maturity, at 36 cm total length.

Interestingly, with some training, visual assessments were found to be slightly more effective than geometric morphometrics, when the sexually dimorphic feature of the supraorbital crest was accounted for. Improvements in visual accuracy occurred as the size of the fish increased following the same correlation as the geometric morphometric methods. This finding underscores the extent of sexual dimorphism between male and female monkeyface pricklebacks. While ultrasound remains the gold standard for accuracy, it can encounter

limitations during the gonadal resting phases, as noted in this study and therefore is not always recommended.

Given the high cost of ultrasound and the robust accuracy of visual and geometric morphometric techniques, aquaculture practitioners could confidently incorporate this research into their broodstock management practices. The cost-effectiveness of the latter two techniques, combined with their decent accuracy rates (70 to 81.4%), makes them viable alternatives to ultrasound. A slight reduction in accuracy may be acceptable considering the substantial cost savings. Concerning the visual identification of sex, practitioners using visual assessment should be aware that this method becomes more reliable as the fish mature and grow larger. Accuracy is lower for smaller monkeyface pricklebacks, but when they reach full maturity (45 cm in length), sexually dimorphic features such as the supraorbital crest become more prominent and are easier to identify. In terms of geometric morphometrics, the most accurate landmarks for distinguishing between sexes are those associated with supraorbital crest and the eye. Therefore, these are the landmarks that I recommend practitioners to focus on.

Future research can build upon the knowledge gained from this study of sexual dimorphism in monkeyface pricklebacks in order to enhance the successful cultivation and management of this species in aquaculture settings.

Chapter 2. First Documentation of Monkeyface Prickleback Reproduction and Embryology in Captivity

RATIONALE AND BACKGROUND

Captive breeding of fish species presents several challenges. A primary bottleneck for breeding is the reproduction process, which can be intricate and species specific. The control and understanding of this process in captivity is essential for the sustainability of commercial aquaculture. Historically, the inability to breed fish in captivity has hampered the progress of domestication of cultured fishes. The development of spawning methods not only leads to success in aquaculture, but can also ease wild take of juveniles or broodstock for aquaculture purposes which would lower the impacts on wild fish populations (Lovatelli et al., 2008).

Understanding and addressing the challenges around gonadal maturation and spawning fish in captivity is typically the first step towards successful hatchery production. Historically, captive spawning has been achieved by a few common methods. One method is to provide an environment in which the fish will reproduce naturally, and another is to induce the fish with hormones treatments via a delivery system (Silva et al., 2008). The use of hormones and artificial insemination has led to major breakthroughs in fish reproduction for aquaculture, allowing for successful breeding of species that were previously difficult to cultivate (Silva et al., 2008). However, in the case for most species new to aquaculture, culturists have historically relied upon gonadal mature or ripe individuals to generate fertilized eggs via natural spawning or strip spawning (Silva et al., 2008). Each spawning approach presents its own difficulties for culturists. Finding fertilized eggs or ripe broodstock from the wild population is logistically difficult and is also sustainably questionable as removal of broodstock consistently can eventually reduce recruitment of larvae via removal of wild embryos or broodstock. Similarly, while more sustainable, achieving volitional spawning requires recreating suitable spawning environments which are generally inhibitive for captive facilities, and likely unreliable due to the vagaries of fish reproduction. Finally, while hormonal

treatments have been effective for some fish, dedicated research is needed for understanding temporal and quantitative dosage regimens to be successful. But in order to breed in captivity, one has to start somewhere using one or a combination of experimental spawning methods.

Understanding reproduction characteristics and early life history characteristics is critical to inform research design. Prior to this study, captive spawning and rearing had not been achieved in captivity for monkeyface pricklebacks, and reproductive and early life history characteristics are mostly unknown. Limited observations of monkeyface eggs have been documented, denoting that the species is a demersal egg layer with an egg size of 2.7 mm, with eggs that are adhesive to rocky substrates (Fitch et al., 1971; Miller and Marshall, 1987; Marshall and Echeverria, 1992). The spawning period occurs between January to August and fish length at post-larval recruitment to the intertidal zone is in the ranges of 1.7-2.2 cm (Miller and Marshall, 1987; Marshall and Echeverria, 1992). Aside from these findings, large knowledge gaps still remain. Most of the early life characteristics (e.g., egg incubation timeline, size at hatching, size and shape of yolk, oil globule, date post hatch of first feeding, etc.) remain unknown for the monkeyface prickleback, mainly due to lack of captive spawning research.

Early life history characteristics range from viviparous fish that produce fully developed juveniles directly after parturition, to fish with long planktonic larval periods of up to 2 years as seen in the freshwater eel (Kamler, 2012). Fish are extremely sensitive during this larval period, with high mortality being commonplace in the wild where typically low percentages of individuals survive from fertilized eggs to the juvenile stage (Hjort, 1914). While high fecundity and low survival are common life history trade-offs, the application of hatchery aquaculture techniques seeks to remove stressors and increase environmental uniformity to overcome these high mortality rates by providing adequate food and eliminating predation, in hopes of maximizing larval survival (Waples, 1999; Fuiman and Werner, 2009). Understanding the early life history and advancing the culture practices based on these characteristics of an animal is crucial for hatching fish eggs and rearing fish through this delicate time.

The length at hatching, larval locomotion, and time of first feeding are extremely important in aquaculture for understanding larval culture requirements. These requirements include when egg

incubation should transition to larviculture, when planktonic live feeds should be incorporated, and what size drain mesh can be used in rearing tanks (Houde, 1972; Huet and Timmermans, 1986; Divanach P., Kentouri M., 2000; Fuiman and Werner, 2009). Other characteristics like yolk depletion, and the time at which developing fish consume embryological energy reserves also directly informs farmers when live feeds must be incorporated (Ostrowski and Laidley, 2001; Ma et al., 2012). This concept is referred to as the time at first feeding, which like other early life history characteristics, is quite variable across species (Kamler, 2012).

The lack of published literature and understanding of the spawning and early life history of monkeyface pricklebacks, despite its potential as a candidate species for aquaculture development, necessitates further investigation. To determine the species' suitability for culture, it is crucial to study the aspects of spawning and early life history within an aquaculture setting. Therefore, this study aims to conduct two spawning reproduction studies over a period of two years, with the objective of gathering valuable insights into the spawning and early life history of monkeyface pricklebacks.

MATERIALS AND METHODS

Two different monkeyface prickleback spawning experiments were conducted with the goal to achieve the first observation of captive reproduction in the species. As mentioned above, limited information exists on monkeyface prickleback reproduction and wild spawning, so this study focused on mimicking natural habitat to facilitate volitional spawning.

Sex Determination from Ultrasound

At the beginning of the reproduction study, I determined the sex of fish using ultrasound techniques. Fish were placed on their right side and a GE Venue 40 portable ultrasound machine with GE L8-18i transducer probe was pressed on the fish’s left abdominal region in order to locate an opaque mass that corresponded with the presence of a mature ovary (Figure 2, Chapter 1). Sex identification using this

technique was based on observations of an ovary (females) in mature sized fish, while males were identified by the absence of the ovary. The accuracy of this method for sex determination was verified by paring ultrasound observations to physical dissections of gonadal tissue, which were also compared to geometric morphometric methods in Chapter 1. After sex identification using the ultrasound, fish were measured, weighed, and Passive Integrated Transponder (PIT) tagged using Biomark (APT12 FDX-B) 12 mm internal tags.

Reproduction Experiment #1

This first reproductive experiment tested spawning substrates and took place in the winter/spring of 2021, to assess whether habitat influences spawning behavior. This time period was chosen because it is the natural spawning season based on gonadal maturity, as the reproductive window occurs between the months of January to August, with peak spawning in February and April (Marshall and Echeverria, 1992). Conveniently, a captive environment with substrate can be easily simulated to mimic a monkeyface prickleback's natural habitat. Monkeyface pricklebacks dwell in rock caves and crevices in the shallow rocky intertidal, and are highly restricted in their movements (Ralston and Horn, 1986). Their shallow rocky cave dwellings were re-created for this experiment by arranging cinder blocks inside the tanks to mimic natural rock caves. However, without knowing what kind of cave is suitable for reproduction, the experiment focused on testing what kinds of environmental substrates are suitable for reproduction by using two types of substrates fixed inside hard cinderblock caves. Chosen substrates were based on the ecology and habitat where monkeyface pricklebacks reside in nature. In the wild, rocky substrate can vary in rugosity, which has been noted to influence other species of demersal egg-laying fish in the wild (Gladstone, 2007).

To test spawning substrates, an array of 9 tanks with flow-through seawater was constructed at the MLML Aquaculture Center. Water temperatures average 14°C at the MLML intakes, which is suitable because monkeyface pricklebacks tolerate 9-25°C. The MLML seawater intake continuously pumps 1300 liters/min of water from 0.4 kilometers offshore, constantly providing nutrients and

environmental conditions that the fish would otherwise experience in the wild. Each tank was 2 m in diameter, creating a shallow standardized 800 liter captive environment. Each environment was covered with shade covers to mimic the light environment in the intertidal created by rocky crevice and seaweeds. Two nesting habitats with different rugosity (rough and smooth) were then secured inside standardized cinderblock caves (**Figure 7**), with sufficient "nesting habitat" of each type for every female to select from. This was designed to test whether monkeyface pricklebacks have certain habitat criteria for reproduction and nesting.

In February 2021, male and female fish were first placed in groups of n = 8 individuals per tank with 4 rough substrates and 4 smooth substrates in each tank, with 9 replicate tanks per treatment (**Figure 7a**). The rough substrates were created by placing small pebbles in a mortar matrix on the inside surfaces of the cinderblock cave (**Figure 7b**). The smooth substrates were created by using mortar without pebbles. The substrates were placed into the tanks and allowed to season for 2 weeks prior to introducing the fish. A sex ratio of 1:1 (4 male and 4 female fish per tank) was initially used because of possible monogamous pair mating, with nest guarding most likely provided by one or both parents. After size measurements, ultrasound determination of sex, and PIT tagging, fish were randomly sorted by size, using only mature fish above the length of maturation (≥ 36 cm) (Marshall and Echeverria, 1992). This first reproduction experiment started on February 17, 2021 and continued until June 14, 2021.

Diet Enhancement and Hormonal Induction for Spawning

Diet is a major influence on reproductive success. Without a proper diet, it is unlikely that fish will develop and mature eggs for spawning (Izquierdo et al., 2001). It is vital that broodstock nutrition is optimized to ensure good larval survival and early development since egg quality is dependent on macro and micronutrients, which is concurrently dependent on nutrient delivery from the female (Izquierdo et al., 2001). Because of this concern, directly after fish collection, all fish in the experiment were fed a mixed algal diet of green seaweed *Ulva*, and red seaweeds *Develerea mollis, Pyropia* spp., and *Mazzaella* spp., using feedstock that was either cultured at the MLML by Monterey Bay Seaweeds using tumble

culture techniques, or collected from the wild. This mix of algae accounts for most of the preferred diet of monkeyface pricklebacks based on published dietary studies and contains protein, lipids, and nutrients necessary for growth and maintenance (Edwards and Horn, 1982; Horn et al., 1982). Feeding 0.20 kg/individual of each algal species per week provided sufficient feed for the captive population based on repeated observations of active feeding as well as uneaten algae left over after feeding.

This study also attempted another strategy for spawning in captivity by chemically inducing spawning for male and female fish by injecting them with Ovaplant-L, a GnRH gonadotropin that chemically triggers the processes of gonad development and spawning (Johnson, 2021). Ovaplant-L was injected into the dorsal musculature at a concentration of 20 µg/kg for fish in 5 of the 9 tanks on April 23, 2021, leaving 4 tanks as an experimental control.

Before and after injection, weekly scope camera egg surveys were conducted using Teslong NTS430 Industrial Endoscope Camera, which had a flexible waterproof borescope that was able to inspect any spawning behavior of all tanks were and to monitor progress. Upon completion of the first spawning experiment on June 14, 2021, male and female fish were then separated by sex and cared for until initiating spawning trial #2 on January 7, 2022.

Reproduction Experiment #2

The second spawning experiment took place during the winter/spring of 2022. This reproduction experiment manipulated sex ratios in broodstock tanks to test whether the sex ratio for males in each tank influenced spawning success. Treatments ranged from highly female-biased sex ratios (1 male : 3 females) to slightly male biased sex ratios (2 males : 1 female), with two treatments in between (1 male : 2 females, and 1 male : 1 female). This experiment used an array of 16 tanks (4 treatments with 4 replicates each) and manipulated sex ratios among treatments to create different densities of male and female fish (**Figure 8**).

Reproductive metrics of egg release, nest guarding, fecundity, and egg size were monitored weekly. Weekly underwater scope camera surveys of all tanks were performed to observe spawning

within treatment tanks as well as to record any preference of artificial habitat type for spawning. Fecundity was documented for successful spawn using Equation (1) to calculate egg volume and Equation (2) to calculate fecundity (i.e., the number of eggs per clutch).

$$Egg\ volume = \frac{\left(\frac{4}{3}\right)\times\pi\times r^3}{1000} \quad \text{[Eq. 1]}$$

$$Fecundity = \frac{egg\ volume}{egg\ mass\ volume} \quad \text{[Eq. 2]}$$

Diet Enhancement and Hormonal Induction for Spawning

Diet enhancement remained the same by providing each fish with a variety of algae consistent with reproduction experiment #1. Similarly, hormonal injections were used to test whether spawning in captivity could be enhanced through chemical induction with a higher dose of Ovaplant-L. For this experiment, I injected the highest recommended dose of Ovaplant-L into the dorsal musculature at a concentration of 100 µg/kg for fish (Johnson, 2021) in 8 of the 16 tanks on April 23, 2021, leaving 2 tanks in each treatment as an experimental control. Weekly egg surveys of all treatments were conducted using a scope camera, which made it possible to inspect all areas of the habitat and to record any spawning activity. After completion of the second spawning experiment on June 14, 2022, male and female fish were then separated by sex, humanely euthanized, dissected, and biologically sampled to confirm sex for ultrasound and morphometric accuracy as described in Chapter 1.

Embryology

Successful spawning events provided opportunities to test methods for egg incubation and sampling of fertilized eggs to track and categorize the stages and timing of embryological development. Fertilized eggs were transferred from the guarding parent into 7.5-liter egg incubation buckets. Egg incubators were placed on a flow-through system with aeration from an air stone, providing oxygenated

water for the eggs. Incubation buckets were held in a water bath that was maintained by aquarium heaters and chillers to keep the temperature consistently at 13°C.

Sampling of eggs for embryological development occurred daily. Eggs (n=5 eggs/day) were carefully removed from the egg mass and used to describe the process of embryological development and growth. The total embryo length, volume of the yolk sac, volume of oil globule, as well as other important embryological development milestones were recorded. All embryological and larval characteristics were observed and measured using a calibrated Leica EZ 4 stereo dissecting microscope equipped with a microscope camera and measuring LAS EZ software Yolk and oil globules were measured following the methods of Blaxter and Hempel (1966) and Cetta and Capuzzo (1982), and yolk volume was calculated as a prolate spheroid (Equation 3), where L is the length in millimeters and H is the height of the yolk sac in millimeters. Oil globule volume was calculated as a sphere (Equation 4) where r represents the radius of the oil globule in millimeters.

$$Yolk\ Volume = \pi/6LH^2 \quad \text{[Eq. 3]}$$

$$Oil\ Globule\ Volume = \frac{4}{3}\pi r^3 \quad \text{[Eq. 4]}$$

Other observations included the timing of key development milestones, such as eye pigmentation, the first observation of a heartbeat, and hatching, etc. Sampling of embryos required using de-chlorination methods to remove the opaque chorion, which involved gently peeling off the chorion envelope and extracting the in-tact live embryos. Live embryos lie on their side and rest within a cylindrical yolk sac (Henn and Braunbeck, 2011). Embryos were placed in *Natural pH*, a pH neutral crystalline gel for immobilization during observations and photographs. All photos for observations and measurements were captured at different calibrated magnifications, ranging from 2.0X-4.2X (depending on total length of embryo) using Leica microscope cameras and Leica Microsystems LAS EZ.Ink software. After hatching, larvae were sampled daily, noting larval growth and development.

Live L-type rotifers (*Brachionus plicatilis*) and Instar I staged nauplii from *Artemia* sp. cysts were batch cultured using culture methods outlined by Dhont and Van Stappen (2003) and Kailasam et

al. (2015) and used as live feeds for larval growth experiments. While larvae accepted these feeds, early larval mortalities stunted further investigation of larval growth and development. Upon 99.99% larval mortality, a single larva remained in the culture system until 18 DPH. The culture system consisted of slow drip flow-through seawater into a 1000 µm screened 7.5-liter incubation bucket, where larvae were fed *Artemia* sp. daily, and *Brachionus plicatilis* hourly using peristaltic pumps. Quick decomposition of larval tissue prevented any description of growth and development upon reaching18 DPH.

RESULTS

This project tested a combination of volitional and hormonal treatment methods to illicit gamete release and fertilization. Although neither approach yielded consistent spawning, the study successfully documented sporadic isolated instances of successful fertilization and parental care of egg masses in captivity. This observation marks the first reported captive spawning in the species.

Reproduction Experiment #1

Throughout the study, 15 different spawning events were observed. Eight events were observed in April and seven were observed in May of 2022. All eggs were collected and examined with microscopy and were determined to be unfertilized (**Figure 9a**). This was determined by several factors. The released eggs were dispersed sporadically throughout the tank (**Figure 9b**) and not clustered in a large adhesive clutch. This is atypical for this type of demersal spawner that is known to exhibit parental care of egg masses. The dispersed eggs did not exhibit an adhesive chorion, which is another indicator of unfertilized eggs. Lastly, eggs were collected and incubated to observe any indications of embryonic development. All eggs collected showed no development when incubated for three days.

This first reproduction experiment did not experience successful fertilization of eggs or other new behavior following GnRH injection because all spawns in both hormonal treatment and control tanks resulted in unfertilized eggs.

Reproduction Experiment #2

Throughout the study, seven spawning events were observed. Five spawning events resulted in unfertilized eggs and were classified as unsuccessful similar to reproduction experiment #1. Only two separate successful spawning events occurred on 5/8/2022 and 5/19/2022, consisting of fertilized egg masses and parental care of those clutches. Both spawning events occurred in separate treatment tanks with no male-male inter-tank interaction. (i.e., only 1 male present in the tank). In each successful spawning event, the male fish guarded the egg mass and displayed parental egg-fanning behavior.

This successful spawning event on 5/8/2022 occurred in treatment #1 (1 male TL = 58 cm: 1 female TL = 54 cm). Nesting and parental care were observed by the male fish coiled around the eggs. Parental care was visually observed and documented, where the male fish displayed behaviors of fanning of eggs using its caudal fin and tail wrapped around the eggs (**Figure 10a**). The eggs were light-yellow in color, small, and not adhesive to substrate, but adhesive to other eggs within the mass (**Figure 10b**). The average egg diameter for this spawn was 1.52 ± 0.10 mm. The eggs were removed from the tank the following day to assess fertilization success and to test egg incubation techniques. Volumetric calculation of the egg mass resulted in an estimated fecundity of 134,434 eggs for this clutch.

The spawning event on 5/19/2022 occurred in treatment #3 (1 male TL = 65 cm: 3 females TL = 43.0 cm, 46.1 cm, 52.5 cm). Nesting and parental care was also observed with the male fish found coiled around the eggs. Male parental care was observed but not photographed. This male was observed to fan and guard the fertilized eggs outside of the cinderblock cave before eggs were removed from the guarding male and tank. The eggs were again observed to not be adhesive to substrates in the tank, but adhesive to other eggs. The average egg diameter in this clutch was 1.61 ± 0.08cm. Volumetric calculation provided an estimated fecundity of 97,753 eggs for this clutch.

Embryology

Fertilized eggs were spherical in shape with 6 adhesive pads in a cruciate pattern (**Figure 11**). Average egg diameter for all eggs measured 1.62 ± 0.11 mm (n = 115). On day 1 after fertilization, commonly referred to as date-post fertilization (1 DPF), the opaque chorion was fully developed encircling the eggs. The spherical yolk was situated centrally and only one oil globule was observed. All egg characteristics are summarized in **Table 2**. Many major milestones of embryonic development were imaged and recorded in this study. These embryological developmental milestones are summarized in **Table 3.** Embryos continued to develop up to 22 DPF, with first hatching on 23 DPF at 13°C.

Gastrula Period (1-4 DPF)

This study was unable to document the first cleavage and blastula period, however the gastrulation period was marked by 3 different stages, the 50% epiboly stage (1 DPF), where the blastoderm creates a dome shaped cell mass over the yolk cell, covering 50% of the animal pole (**Figure 12a**). This stage is named by the fraction of the embryo that the blastoderm has spread across (i.e., the percent-epiboly). The shield stage occurred at 3 DPF, where the blastoderm is still 50% epiboly but there is a thickening of the germ ring, called the embryonic shield on the dorsal side of the embryo (**Figure 12b**). Lastly in the gastrula period is the bud stage (4 DPH), which occurs when there is 100% epiboly. At this stage the epiboly is fully closed and creates a distinct delineation between the posterior epiboly and the yolk. Near the posterior end of the axis there is a distinct swelling of the tail bud (**Figure 12c**). On the opposite end, anterior to the tail bud, another prominent thickening of the embryo clearly marks where the head develops (**Figure 12c**). This stage is important because it helps define the axis of the embryo.

Segmentation Period (5-9 DPF)

The are many morphological developments during segmentation, including key milestones that were observed such as V-shaped somite development, the development of the optic and Kupfer's vesicle, the hindbrain, otolith formation, and tail development. 5 DPF captures the 8-somite stage. During this

stage the first 8 somites are clearly visible. Somites are precursor cells that give rise to important structures for invertebrate body plans, and many of these furrowing marks will eventually develop into musculature, originating from the trunk of the fish (**Figure 13a**). During this period, the first development of the optic vesicle (precursor of the eye) was observed. 6 DPF marks the first observation of hindbrain development and the beginning developments of the Kupfer's vesicle (**Figure 13b**). The Kupfer's vesicle, while temporary, helps organize the left from the right side of the animal during development. At DPF 7, further development of the optic vesicle, Kupfer's vesicle, and the beginning of tail development occurred (**Figure 13c**). By 8 DPF, the tail becomes well extended from the yolk and there are observable divisions of the hindbrain where there is a hollowed-out area of the otic vesicle (**Figure 13d**). This otic vesicle is adjacent to where the otoliths will begin to develop. At this stage, somites and the notochord continue developing, exhibiting a characteristic stack of pennies appearance along the trunk of the embryo (**Figure 13d**). 9 DPF is characterized by muscle development along the notochord along with spontaneous myotomal contractions that produce the first twitching and lashing from side to side of the embryo. At this stage, the tail is well extended and the first observation of the otoliths and development of the anus and intestine were recorded (**Figure 13e**).

Pharyngula Period (10-22 DPF)

The term pharyngula refers to an embryo that has developed a classic vertebrate body plan (Gould 1977). It is also the time of development where the embryo is developing in length, evident by the straightened head and tail, developing mouth, eyes, pectoral fins, and other general features. There are several important developments during the pharyngula period outlined below.

The fins first began to form on 10 DPF (**Figure 14a**), which were barely observable at the beginning of the period. Throughout this stage, the fins become more prominent whereby they develop collagenous fin rays called actinotrichia **(Figure 14b and 14c**). Pigment cells first begin to differentiate as seen by the pigmented retinal epithelium (outer eye) on 9 DPF (**Figure 15b**). The eyes continue to pigment and develop from 9 DPF up until hatching 23 DPF (**Figure 15d**). More pigmentation occurs

around the embryo slowly and by 20 DPH melanophores appear in asterisk patterns on the intestine (**Figure 15c**). Pigmentation also forms well-defined longitudinal stripes on the tail and caudal fin by 20 DPF (**Figure 15d**).

The circulatory system began to form starting 9 DPF. The cone shaped heart began to beat, and then formed well-delineated chambers by the time of hatching. At 9 DPF, the heart was visible as a cone-shaped tube directly behind the eye (**Figure 16a**). Early in this stage the gut tract began to form (9 DPF), while also developing the pronephric duct. The pronephric duct is a channel for kidney waste and opening to the egg cytoplasm just posterior to the anus. The duct itself is difficult to visualize during development but above this and posterior to this duct is the "Blood Island" which consists of the majority of the first dividing and differentiating red blood cells and is clearly visible at 14 DPF (Kimmel et al., 1995) (**Figure 16b**). These blood cells eventually moved more anteriorly, and many blood cells also occupied spaces around the yolk ball (18 DPF) (**Figure 16c**) becoming key parts of the circulatory system.

9-10 DPF anal and caudal membranes began to form the beginning of the larval fins. As the embryo developed, the frequent spontaneous lashing activity became less common, but any light touch excited a reflexive wriggle. The pectoral fins began development on 16 DPF and finished development and were fully formed by 21-22 DPF (**Figure 17a**). Behavioral development was observed during this stage, including locomotion of the tail leading to swimming ability before hatching, in conjunction with the mouth also becoming fully developed before hatching on 22 DPF (**Figure 17b**).

Hatching and Larval Development (23-30 DPF)

Hatching of exterior eggs first began on 23 DPF and lasted until 31 DPF. Just prior to hatching, the chorion was observed to degrade, allowing the embryo to easily break through the remaining thin membrane (**Figure 17c**). A newly hatched larva is depicted in **Figure 17d**. The minimum length at hatching was 6.82 mm, while the maximum length at hatching was 8.26 mm. The average size at hatching was 7.43 ± 0.72 mm. Sequential hatching was observed, such that eggs situated near the periphery of the egg mass hatched before eggs near the center of the mass. The hatching period lasted for 8 days, with 2

days where no hatching occurred, all of which suggests that eggs near the center of the mass developed slower with up to 31 DPF until hatching. Because of high mortality, only 108 eggs hatched, (5/31/22 = 2 hatched, 6/1/22 = 7 hatched, 6/2/22 = 25 hatched, 6/3/22 = 0 hatched, 6/4/22 = 16 hatched, 6/5/22 = 0 hatched, 6/6/22 = 48 hatched, 6/7/22 = 10 hatched) limiting sample size of measured hatch to n = 20 across all hatching days. The low sample size limited correlation analysis between timing of hatching and hatch size. Poor hatch rate was due to an unidentified fungus that infected the second egg mass and caused high mortalities as well, with an equally poor hatch rate (**Figure 18**). For hatched larvae, the yolk sac was fully depleted at 2 Days Post Hatch (DPH). For this study, yolk sac and oil globule utilization were characterized over embryological development and growth and is illustrated in **Figure 19**. The results from the quantification of yolk and oil globule consumption suggest that yolk sac reserves are utilized first by the developing embryos and were mostly utilized in the first stages of embryological development (1 DPF – 10 DPF). In the first 10 days of development the yolk reserves were depleted to 9.8% remaining of its original volume, while the oil globule volume still remained at 50% of its original volume on 10 DPF. The oil globule became the main energy source throughout the rest of embryological development depleting gradually, and at time of hatching the oil globule consisted of 2.97% +/- 3.5% of its original volume while the yolk reserve consisted of 0.57% +/- 1% of its original volume.

The embryological timeline is depicted in **Figure 20**. This timeline consists of photographs taken throughout embryological development, which can be helpful for visualization of growth, yolk and oil globule utilization and other major developmental milestones.

First Feeding

First feeding was observed on 1 DPH, with an observation of rotifer (*Brachionus plicatilis*, L-type, average size of 239 μm) ingestion, evident by the presence of green rotifers within the larval stomach (**Figure 21a**). Rotifers were fed at a density of ~10 count/ml, while *Artemia* was fed at a density of ~3 count/ml. Food densities were calculated by volume and confirmed by water sample counts taken carefully from larval incubation buckets. The next major milestone for larva culture was the time post-

hatch where larvae can eat larger food like *Artemia* (instar I nauplii 300-500 µm), which occurred at 3 DPH in monkeyface pricklebacks (**Figure 21b**). Active hunting of food was recorded on video directly after feeding. High larval mortality and the destructive nature of larval development sampling prevented sampling beyond 3 DPH, but the few remaining larvae were cultured until the final larvae had succumbed to mortality reaching 18 DPH.

DISCUSSION

Limited understanding of spawning in captivity, embryo development, and the crucial transition from endogenous to exogenous feeding can impede the sustainable growth of aquaculture practices. Spawning success plays a pivotal role in ensuring the reproductive efficacy and long-term sustainability of aquaculture operations (Silva et al., 2008). However, several species exhibit intricate social behaviors (such as male-male competition), requirements for certain nutritional items, photoperiod and light intensity, temperature, salinity or other environmental conditions, or specific nesting and spawning substrates – all of which can present formidable challenges for achieving successful spawning in captivity (Budd et al., 2015). This study was the first to successfully to breed monkeyface pricklebacks in an aquaculture setting and despite some progress, it encountered a number of challenges in achieving successful and consistent spawning under captive conditions.

Out of the 72 broodstock fish utilized in this experiment, only two pairs managed to successfully spawn and fertilize egg masses in captivity. While these results may not be immediately applicable to commercial culture, they provide valuable insights into the reproductive dynamics. Notably, in reproduction experiment #1, it was observed that females were producing and releasing eggs, yet the male fish failed to fertilize them, resulting in dispersed and unfertilized eggs scattered around the tank. This observation suggests that male fish may be influenced by the presence of other males within the same tank, leading to decreased spawning success. In the realm of aquaculture, instances where males hinder

successful spawning are relatively rare, as most spawning limitation stems from a lack of female participation or mature gonads (Bromage et al., 1992; Mylonas et al., 2010).

The hypothesis that male-male interactions are one factor responsible for reducing spawning success in captivity has been reported in the literature, although somewhat rarely. For instance, in studies of Nile tilapia, rainbow trout, and European bitterling, it was reported that when multiple males were present in the spawning area, a higher frequency of scattered eggs occurred, indicating intense competition among males for fertilization opportunities and nesting sites (Reichard et al., 2004; Martin-Smith, K. M and Armstrong, M. J., 2008; Mustafa, S et al., 2019). Furthermore, the rainbow trout and European bitterling studies have shown that when there are more males competing for a limited number of nesting sites, the males become more aggressive towards each other, leading to higher rates of physical conflict and injury. This can result in reduced survival and growth rates, making male-male competition an important consideration in rainbow trout aquaculture (Martin-Smith and Armstrong, 2008; Reichard et al., 2004). Male-male competition was observed in the reproduction treatment tanks, and while not widespread across all tanks, the tanks with higher densities of male fish consistently had issues with fighting (distinct bite marks, or injuries to male fish). These examples underscore the significance of male-male competition as a potential factor influencing spawning success in various fish species. Similarly, on top of the occasional bite marks, the many observations of scattered eggs in both reproduction experiments appeared to occur in only the tanks with higher densities of males, which can lead a culturist to conclude that tanks with high densities of males may be similar to other studies where male-male interactions contribute to low spawning success.

Fish behavior beyond antagonistic male-male interactions may also have influenced the spawning success of monkeyface pricklebacks in this study. In particular, fish courtship is known to play a crucial role in the reproductive success of various species (Johannessen et al., 1993; Fleming and Huntingford, 2012; Vacco et al., 2021; Ahamed and Tokumoto, 2023), and it is likely to have been a key factor in the spawning of monkeyface pricklebacks. The courtship behavior of wolf eels, a related species, serves as an illustrative example of fish courtship in aquaculture. In captivity, wolf eels have been observed engaging

in courtship and pairing up to six weeks before spawning (Marliave, 1987). This courtship behavior involves males butting their heads against the female's abdomen, providing a clear visual cue (Marliave, 1987). However, understanding courtship dynamics in monkeyface pricklebacks is more challenging due to their complex captive observations. In the initial reproduction experiment, it was common to observe multiple individuals (3-4) residing in the same cave substrate, making it difficult to determine when pairing occurred. In the second experiment, no observations of courtship were seen as monkeyface pricklebacks are most active at night (Ralston and Horn, 1986). Monkeyface pricklebacks were paired up for 12 weeks prior to their reported peak spawning period of March-May (Marshall and Echeverria, 1992), while this is twice the amount of time than the wolf eel study (6 weeks) it is unclear if 12 weeks is sufficient given their sedentary nature. There may also be an aspect of mate choice that influences monkeyface prickleback courtship. In this study the first successful spawning occurred with 1 male TL= 58cm : 1 female TL=54cm. The second spawning occurred with an extremely large male (TL = 65 cm) and a female that was either TL = 43.0 cm, 46.1 cm, or 52.5 cm. While inconclusive, this result may be the first indication that monkeyface pricklebacks are potentially positive assortative maters (larger males paired with larger females), as seen in related fish such as the high cockscomb *Anoplarchus purpurescens* (Coleman, 1992). This highlights the need for further research into mate choice and courtship behavior in monkeyface pricklebacks. Investigating the specific cues and behaviors involved in their courtship rituals could provide valuable insights into monkeyface eel reproductive biology. By identifying the courtship behaviors and signals that precede successful spawning, researchers can enhance the understanding of monkeyface prickleback reproduction and potentially develop more reliable methods for egg production. Expanding knowledge in this area will be essential for aquaculture efforts of this species.

However, in the case of *Anarhichas lupus,* researchers suggest other spawning methods that each have their own way to deal with this fish’s complex courtship behavior and facilitate successful reproduction in captivity (Johannessen et al., 1993). These authors report that spawning behavior in females and low fecundity in males resulted in unsuccessful or limited success in pairing due to the complex nature of their courtship and reproduction. In their study females initiate courting behavior and

seem “restless” around a chosen mate, while males remain passive in courtship. During reproduction males they exhibit side-bending” or stretching and bending repeatedly around a female while the female exhibits “side resting” around the bending male, which is thought to be related to the act of reproduction. Therefore, they suggested two alternative methods for successful reproduction. The first being mass volitional spawning in a large enclosure, where 90 fish were kept in tanks with large bottom basins with 1500 m^2 of surface area. In this attempt, 20 females spawned, but unfortunately while there was plenty of space and habitat, other eager fish ate many of the eggs. While this technique was “successful” in the sense 20 females spawned, the unknown egg predation factor in these methods resulted in it not being recommended. The other successful techniques are different methods for artificial fertilization, where researchers either strip spawned the eggs and milt, or performed artificial insemination of milt through the body wall or oviduct of the female. While strip spawning *Anarhichas lupus* had varying degrees of success, artificial insemination of the female obtained close to 100% fertilization of eggs. Further investigation and understanding of artificial fertilization such as strip spawning may be the key to unlocking successful spawning methods for monkeyface pricklebacks in the future.

Additionally, the study revealed significant insights into male parental care exhibited by the species. In both instances of successful spawning during reproduction experiment #2, the male monkeyface pricklebacks were observed diligently guarding the egg mass and engaging in fanning and care of the egg mass. Previously it was unknown which parent takes on the responsibility of egg guarding (Lea and Reilly, 2001). The identification of male parental care in monkeyface pricklebacks is consistent with the findings that Stichaeidae are one of the few families known to contain species with either male-only or female-only parental care, but no species with biparental care. Male parental care has been documented in species like *Xiphister mucos* and *Xiphister atropurpureus* by Marliave and DeMartini (1977). While female care has been documented in species like *Anoplarchus purpurescens* (Coleman, 1992). Understanding the prevalence of parental care type in the Stitchaidae family contributes to our broader comprehension of reproductive strategies and social dynamics within this taxonomic group.

The presence of male fish actively guarding the egg mass and engaging in fanning behavior serves multiple purposes. First, it ensures the protection of the vulnerable eggs from potential predators and environmental stressors (Boesch, 1992). Secondly, the fanning behavior enhances oxygenation and circulation around the eggs, promoting their development and survival (Wootton, 1998). This is seen in several species of fish such as common gobies *Pomatoschistus microps* (Jones and Reynolds, 1999) and three-spined sticklebacks *Gasterosteus aculeatus* (Reebs et al., 1984). While this activity can be costly in terms of energy, insufficient oxygenation of the eggs can lead to delayed development, malformation, and even death of the offspring (Fonds and Veldhuis, 1973; Kramer, 1987; Hale et al., 2003). The active involvement of male monkeyface pricklebacks in these crucial parental care behaviors emphasizes their significant contribution to reproductive success and offspring survival. By shedding light on the occurrence and significance of male parental care in monkeyface pricklebacks, this study enhances our understanding of the complex reproductive behaviors and strategies in this species. The findings provide valuable insights for conservation efforts, captive breeding programs, and the overall management of monkeyface pricklebacks in both natural and captive environments.

This study yielded several unexpected findings. One intriguing observation was the occurrence of successful spawning outside of the traditional cinderblock cave (spawning event #2). This finding was particularly surprising considering that previous observations of monkeyface prickleback reproduction had exclusively occurred inside rocky intertidal caves. The implication of this observation is that the specific substrate type, such as rocky surfaces, may not be an absolute requirement for successful spawning. This discovery opens up the possibility of using alternative materials such as PVC or other plastics that are lighter and easier to take care of in an aquaculture tank. Another unexpected finding pertained to the adhesion properties of the fertilized eggs found in reproductive study #2. Contrary to a previous report in the literature by Fitch et al. (1971), which suggested that the eggs were adhesive to rocks, this study found that the eggs were adhesive to themselves rather than the substrate. This discrepancy highlights a divergence from the established knowledge regarding the adhesive properties of

monkeyface prickleback eggs. Such deviations emphasize the need for further investigation to fully comprehend the unique characteristics of monkeyface prickleback spawning behavior and reproduction.

While the spawning experiments conducted in this study did not indicate clear associations with sex ratios or habitat type, two successful spawning events generating clutches of fertilized eggs provided the first and valuable opportunity to track embryological development of the species. The embryonic development of monkeyface pricklebacks follows a pattern similar to that of teleosts, with zebrafish being a particularly well-studied model species. Typically, embryonic development milestones are achieved in a similar sequence, however the embryonic ontology timeline for zebrafish is much shorter than for monkeyface pricklebacks (Kimmel et al., 1995). Zebrafish embryonic stages are measured by the hour and embryos take a total of 72 hours to develop and hatch. In contrast, the development of monkeyface prickleback embryos was observed to be much slower (23-30 DPF), which can affect incubation success and directly impact hatching rates. When compared to other marine fish species, such as cabezon *Scorpaenichthys mamoratus* (hatching between 25-45 days at 8-10°C) and lingcod *Ophiodon elongatus* (hatching between 21-38 days), monkeyface pricklebacks have a developmental timeline that is typical of many cold-water species, lasting several weeks (Gregg, 2003; Lauth, 1989). Temperature is a crucial factor that greatly affects the developmental timeline of fish species, and while the influence of temperature on embryological development was not investigated, an average of 13°C during embryogenesis resulted in an incubation of 23 days for the time at first hatching in monkeyface pricklebacks.

During incubation, fish eggs possess two primary energy sources: yolk and oil globules. Yolk serves as the primary energy source, while oil globules provide additional energy and buoyancy (Kamler, 2002). These energy sources are utilized during the incubation period to support the growth and development of the embryo. I examined the difference between yolk depletion and oil globule depletion to relate how each energy source is utilized during different periods of fish egg incubation. For monkeyface pricklebacks, I found that yolk is the primary energy source during the early stages of embryonic development, being mostly utilized within the first ten days of incubation, while oil globules

provide supplementary energy during the later stages until hatching. Monkeyface pricklebacks mostly utilize their yolk reserves first during early development because the yolk provides the large amount of energy needed for early cell divisions as well as development of the heart, brain, and skeletal system. Yolk is rich in proteins, lipids, and carbohydrates, making it an ideal energy source for supporting these processes (Kjørsvik et al., 1990). After the first 10 days of incubation many of these important physiological systems are developed, resulting in less than 10% of the yolk reserve remaining. At this point, monkeyface prickleback embryos turn to utilizing their oil globule reserves for the remainder of development until hatching, when both yolk and oil globule energy reserves are <5%. This sequence of yolk and oil globule utilization is common during embryo development because processes such as cell division, differentiation, and organogenesis require a high demand for energy and nutrients (Kamler, 2002). As the embryo develops and the yolk reserves are gradually depleted, the oil globules become a more significant energy source, providing lipids that can be metabolized to produce energy via beta-oxidation (Kamler, 2005).

Yolk and oil globule utilization varies among fish species and can be influenced by factors such as temperature, oxygen availability, and egg size (Kamler, 2005). For example, in Atlantic cod *Gadus morhua*, yolk depletion occurs rapidly during the first few days of incubation and then slows down until hatching (Kjørsvik et al., 1990). In contrast, in zebrafish *Danio rerio*, yolk depletion occurs at a more constant rate throughout the incubation period (Riddle and Hu, 2021). Oil globule utilization also varies among fish species and is influenced by the same factors such as temperature, egg size, and species-specific metabolic rates (Kamler, 2005). For example, in red drum *Sciaenops ocellatus*, oil globule depletion occurs rapidly during the first few days of incubation and then slows down until hatching (Holt et al., 1981). In contrast, in European sea bass *Dicentrarchius labrax*, oil globule depletion occurs more gradually throughout the incubation period and 30% of the globule remains at first exogenous feeding (Rønnestad et al., 1998). This sequential utilization of energy sources ensures that the developing embryo has access to a continuous supply of energy throughout the incubation period. The differences in yolk and oil globule utilization strategies can be attributed to the specific energy requirements of each

developmental stage and the metabolic pathways involved in utilizing these energy sources (Kamler, 2002).

Several studies have investigated the factors that can influence the hatching rate of marine fish species in aquaculture. Liu et al. (2017) reported that the hatching rate of spotted sea bass *Lateolabrax maculatus* eggs was significantly influenced by water temperature, salinity, and dissolved oxygen levels during incubation. Ranjan et al. (2018) reported that the hatching rate of Indian pompano *Trachinotus mookalee* eggs was significantly affected by the quality of the eggs, the water temperature, and the light intensity during incubation. These studies highlight the importance of optimizing environmental conditions and broodstock management to improve the hatching rate of marine fish species in aquaculture. This study utilized a water temperature of 13℃, which falls within the typical temperature range for monkeyface pricklebacks (Love, 2011). Thus, factors other than water temperature may have contributed to the observed low hatching rate (<1%). One factor that can greatly affect hatching success is bacterial and fungal infections of the egg masses (Olafsen, 2001). Fungal/bacterial growth appeared to overtake parts of the egg masses for both fertilized clutches in this experiment. While a definitive identification of the growth on the embryos was not determined, the outward white fuzzy appearance of the growth seemed consistent with a fungus **(Figure 18**). The use of small incubator tanks with untreated seawater during the incubation period likely resulted in the proliferation of the fungal growth on the embryos and subsequently terminated them. The development of the embryos over a period of three weeks provided ample time for such microorganisms to grow and infect the eggs, leading to a low hatching rate.

In the wild, the occurrence of bacteria and fungus on eggs is unlikely due to the high-water volume and exchange rate, which dilutes any harmful microorganisms present. However, in aquaculture, the disease risk is greater due to the lower volume of water, which increases the concentration of harmful microorganisms and potential for infection (Olafsen, 2001). Therefore, it is necessary to take proper precautions during egg incubation to prevent the proliferation of harmful microorganisms. There are several treatment methods that can be used to prevent the occurrence of bacteria and fungus on eggs

during incubation. One of these methods is the use of formalin, which is a common disinfectant used to kill harmful microorganisms (Francis-Floyd, 1996; Rach et al., 1997). Ozone treatment is another effective method of disinfection that can be used to eliminate harmful microorganisms (Powell and Scolding, 2018). Lastly, leaving eggs with parents who can provide appropriate care prior to hatching can also be an effective method of preventing the occurrence of bacteria and fungus on eggs, as well as parental care as a method that can help to protect the eggs from harmful microorganisms. This technique of allowing the parents to provide care in aquaculture facilities is sometimes used in smaller tilapia hatcheries, which leave eggs naturally to brood in the mouth of the male or female fish (Sagua, 1987). Allowing for parental care during egg incubation in aquaculture is also effective for octopus which exhibit parental cares such as egg fanning (Uriarte et al., 2011).

Although only a limited number of eggs hatched from the study, the timing of hatching was found to be asynchronous over an 8-day period. Asynchronous hatching is seen in both broadcast and demersal marine fish and the hatching period can vary depending on the species. For example, in cod *Gadus morhua*, a 3 day hatching period is thought to be one way to reduce competition for resources among siblings and a way for parents to improve the chances that some of their offspring meet favorable conditions to survive in a variable environment (Politis et al., 2014). Based on this logic, the longer asynchronous hatching period of 8 days for monkeyface prickleback eggs may be related to the fact that eggs typically face a variety of conditions in the intertidal. By having a longer hatching period the chances of favorable intertidal conditions may increase.

Furthermore, asynchronous hatching may also be due to factors such as physical space to hatch or the influence of current velocity and oxygen availability within the center of the developing egg masses. Monkeyface prickleback egg masses are dense, preventing the viability of centrally located eggs hatching early. While it was not conclusively investigated, the 8-day hatching rate in this study suggests that eggs towards the center of the egg mass may develop slower than the ones on the periphery, so that they can be unincumbered by other eggs during hatching. Additionally, slower development of centrally located eggs may also be influenced by the relationship between current velocity and oxygen availability. For example,

Lingcod *Ophiodon elongatus* lay demersal egg masses and embryo development near the center of the egg mass is hindered by low oxygen content from poor water current circulation. In that study, current velocities of 10-15 cm s^{-1} were needed to maintain adequate interstitial oxygen levels in the egg mass (Giorgi and Congleton, 1984). Water flow in monkeyface prickleback egg incubators (while not measured) were anecdotally above the threshold needed for lingcod egg masses, however further investigation of what current velocity is required specifically for monkeyface prickleback eggs masses is necessary because it is unknown if monkeyface prickleback embryos require a higher water velocity than lingcod eggs.

Following embryogenesis and hatching, larvae are released to continue development in the pelagic environment. During this larval period, fish grow and the transition from endogenous to exogenous feeding is another key phase in the early life history of many aquatic species and aquaculture husbandry (Hjort, 1914; Kiørboe et al., 1985). During this stage, larvae shift from relying on their internal yolk reserves to actively feeding on external food sources. However, the lack of knowledge about feeding preferences, and suitable prey items for larvae can impede the successful transition and survival in captivity (Qin et al., 1997). Because monkeyface pricklebacks have depleted most of their endogenous feeding reserves, it is critical that culturists provide live feeds immediately upon hatching to increase the chance of first feeding and survival for the species. The size at hatching, the larval mouth size, and time of first exogenous feeding are also important in fish aquaculture because they can affect a fish's survival, growth rate, and overall health (Anderson, 1988). A larger size at hatching can provide the fish with more energy reserves, which can increase its chances of survival and growth (Garrido et al., 2015). Similarly, an earlier time of first exogenous feeding can provide the fish with necessary nutrients and energy, which can also enhance its growth and survival.

Monkeyface prickleback larvae observed during this study hatched at a size of 7.43 ± 0.72 mm, and first feeding occurred at 2 DPH, with a mouth size of 250 µm. While the hatching size of monkeyface pricklebacks can be slightly larger than some marine species, the small mouth size and short first feeding timeline indicate that the selection and implementation of live feeds are critical for the success of

culturing this species. This study observed that monkeyface prickleback larvae were able to first feed on rotifers (2 DPH) but then quickly transitioned to *Artemia* nauplii (3 DPH). This transition to *Artemia* was rapid compared to many marine fish that consume a rotifer diet between 7-30 days DPH (Fujita, 1973; Lubzens et al., 1989; Treece and Davis, 2000; Imsland et al., 2006). This quick transition to *Artemia* will be beneficial for monkeyface prickleback culture because live feed production is labor intensive; therefore, having a short rotifer feeding period will reduce the effort in producing multiple live feeds.

The importance of larval mouth size in aquaculture is also evident in both marine and freshwater fish species. For example, in the case of the gilthead sea bream *Sparus aurata*, a marine fish species with a relatively small larval mouth size (200-400 μm), careful attention to feeding strategies is required to ensure successful larval rearing (Morretti, 1999; Izquierdo et al., 2001). In contrast, some freshwater fish species have larger larval mouth sizes and can tolerate a wider range of feed types. For instance, the African catfish *Clarias gariepinus*, a species commonly cultured in Africa and parts of Asia, has a relatively large larval mouth size (500-800 μm) and can be fed on artificial diets at an earlier age (Owori-Wadunde and Kityo, 2014). Furthermore, larger hatching sizes and longer periods before first feeding may be beneficial in aquaculture because they allow for greater energy reserves and a longer window for fish to acclimate to their environment before needing to feed (Kamler, 2008). However, delayed feeding can also result in slower growth rates and increased mortality if fish are not provided with the appropriate nutrition at the optimal time (Kestemont et al., 2007). Ultimately, the best approach to rearing fish in aquaculture depends on the specific species and the conditions under which they are raised.

CONCLUSION

This study investigated the key aspects of reproductive function and early life history for the monkeyface prickleback, detailing some of the first reported information concerning impediments to captive spawning, potential broodstock sex ratios, fecundity, parental egg care, embryological development, and early larval development. This first investigation emphasizes the importance of mate

pairing to optimize spawning success and also a comprehensive exploration of larviculture to optimize hatchery techniques for the species within an aquaculture setting. By filling these knowledge gaps, we can make significant contributions to the development of monkeyface pricklebacks as an emerging aquaculture species. Overall, these findings not only contribute to a deeper understanding of monkeyface prickleback spawning behavior, but the unexpected observations reported herein challenge preconceived notions and open new avenues for investigation, potentially enhancing the success of captive breeding programs and development of the species for commercial aquaculture.

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TABLES

Table 1. Statistical analysis of shape in male and female monkeyface pricklebacks.
Procrustes ANOVA – Procrustes multivariate analysis of variance; CVA - Canonical variance analysis; DFA - Discriminant function analysis; SS - Sum square; df - degrees of freedom; D^2 - Mahalanobis distance; T2 - Hotteling's test; All landmarks – All cranial landmarks; Supraorbital crest landmarks – Landmarks #11-25 (blue landmark curve); Eye landmarks- Landmarks (#1, 2, 7, 8, 9, 10).

	Procrustes's ANOVA					CVA					DFA		
Structure	SS	df	F	*P****	Pillai's trace	Eigenvalue	Var (%)	Cum (%)	*p****	D^2	T^2	T^2*p****	*P****
All landmarks	0.1355	46/4508	25.76	<.0001	0.79	3.05	100	100	<.0001	3.46	299	<.0001	<.0001
Supraorbital crest landmarks	0.0717	30/2940	5.96	<.0001	0.5	0.96	100	100	<.0001	1.94	94	<.0001	0.0032
Eye landmarks	0.5358	8/392	49.04	<.0001	0.62	1.29	100	100	<.0001	2.25	127	<.0001	<.0001

Table 2. Egg characteristics

Oil globule	Chorion type	Perivitelline space	Yolk type	Diameter
1 oil globule	Opaque - adhesive to eggs	Narrow	Unsegmented	1.62 mm

Table 3. Embryological and larval development milestones

Stage	DPF	Characteristics
Gastrula	1	50% epiboly; Blastoderm remains uniform across animal pole
Gastrula	2	50% epiboly; Shield is visible from animal pole
Gastrula	4	100% epiboly; Tail bud prominent, first sign of head
Segmentation	5	8-somite stage; Optic vesicle development
Segmentation	6	Hindbrain development
Segmentation	7	Beginning of tail development
Pharyngula	8	Divisions in hindbrain
Pharyngula	9	First heartbeat; first sign of otoliths
Pharyngula	16	Start of pectoral fin development
Pharyngula	19	Pectoral fins developed fully developed
Pharyngula	20	Swimming ability
Pharyngula	22	Mouth fully developed
Hatching	23	Chorion Degrades, Hatching
Laval Period	DPH 1	First Feeding of Rotifers
Larval Period	DPH 2	Yolk sac depleted
Larval Period	DPH3	First Feeding of Artemia

FIGURES

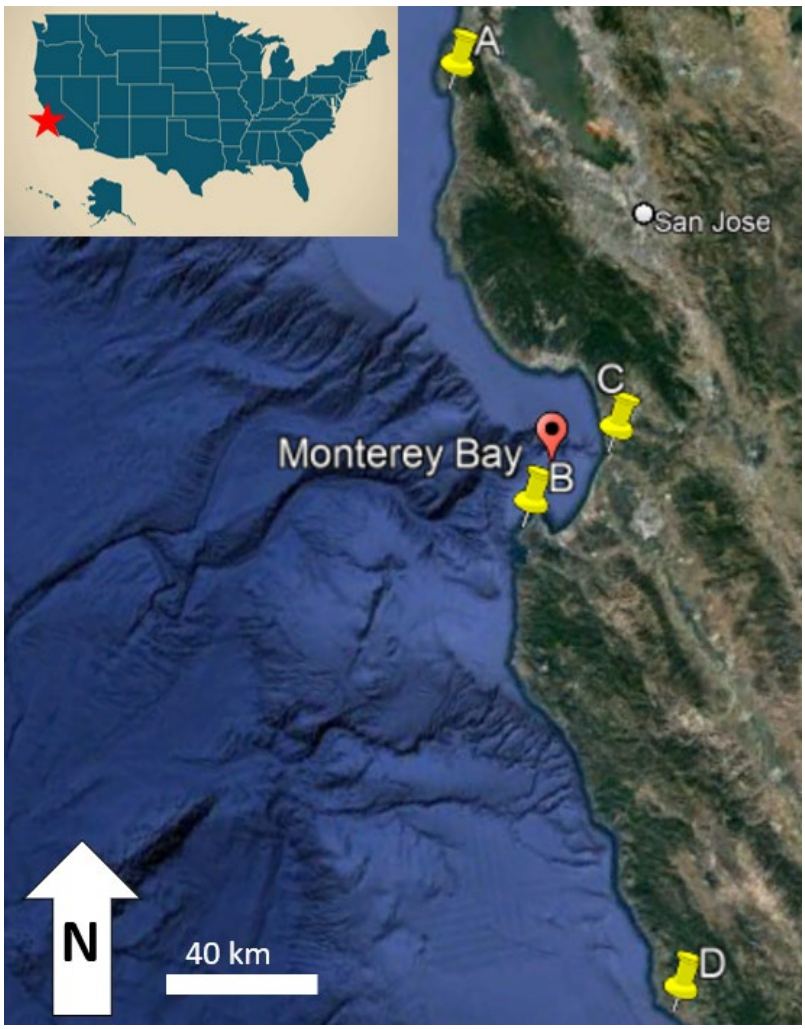

Figure 1. Map displaying monkeyface prickleback collection sites. Pin A) (Half Moon Bay, CA); **Pin B)** (Carmel Coast, CA); **Pin C)** (Moss Landing, CA); Pin D (San Simeon, CA).

Figure 2. Ultrasound imaging of monkeyface prickleback. This image depicts a female monkeyface prickleback (bottom) during an ultrasound scan of the left abdomen of the fish. The granular texture on the ultrasound screen (highlighted by red oval) displays an image of the fish's ovary.

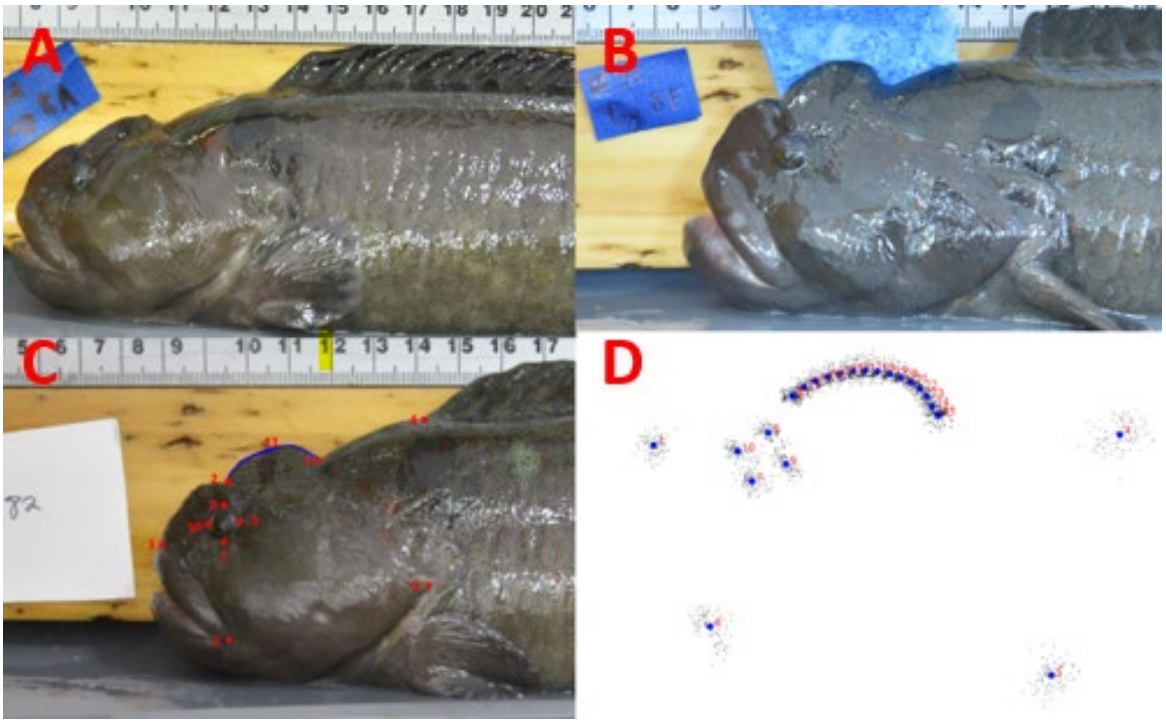

Figure 3. Example photos for morphometric analysis with digitized landmark placements: **A)** Image of female monkeyface prickleback. **B**) image of male monkeyface prickleback (note larger head crest). **C**) Example photo of image with digitized landmark placements. This photograph is an example image displaying the points on the fish that were used as landmarks for analysis. (1) tip of snout, (2) anterior end of supraorbital crest, (3) posterior end of supraorbital crest, (4) dorsal fin insertion, (5) tip of opercula, (6) tip of left facing maxilla, (7) bottom of eye (8) top of eye, (9) posterior of eye, (10) anterior of eye, (11) blue landmark curve on following edge of supraorbital crest. **D**) Graphical output of Procrustes coordinates for all landmarks after Procrustes superimposition.

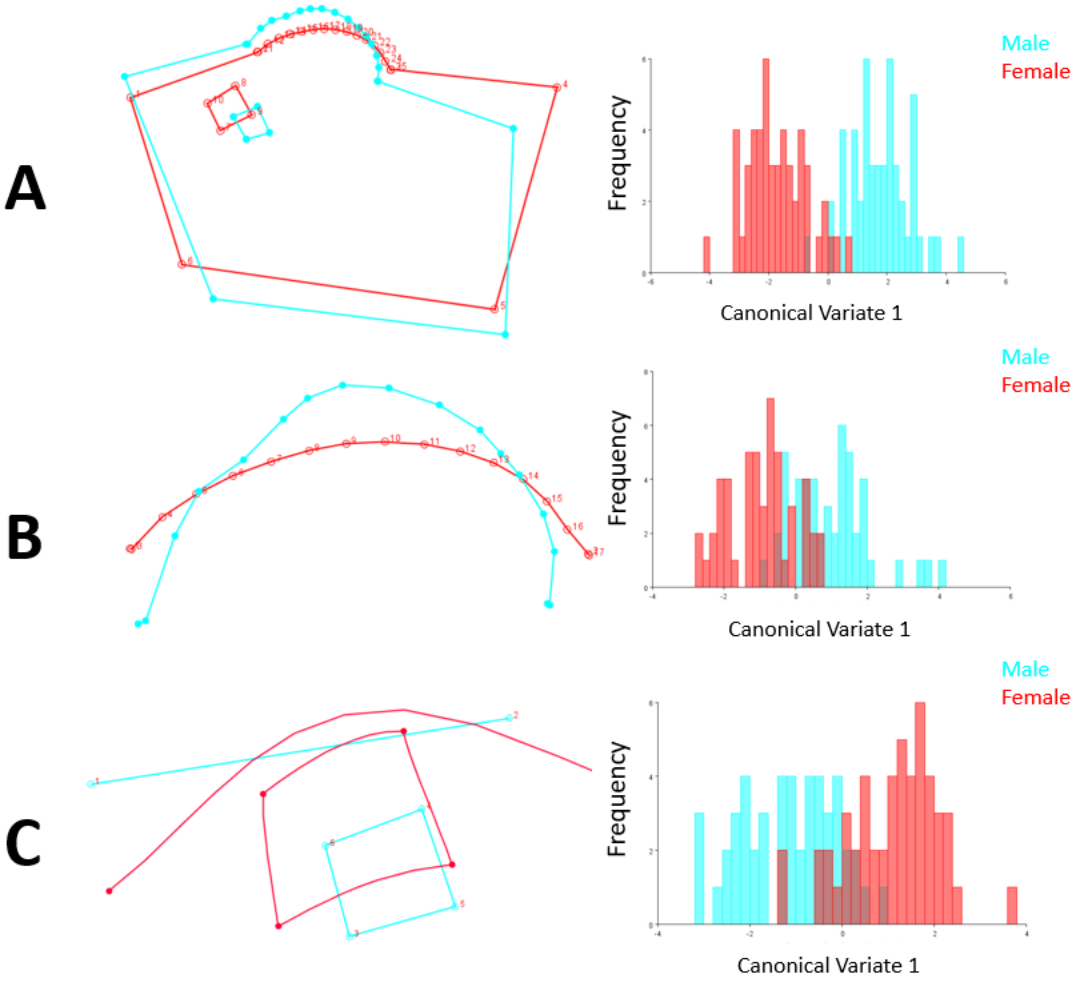

Figure 4. Canonical Variate Analysis (CVA) graphical outputs using wire frame graphs and histograms. This CVA shows how all landmarks, when grouped by sex vary in size and shape. **A)** Wire frame graph and Canonical Variate 1(CV1) frequency histogram for all head landmarks. **B)** A wire frame graph of and CV1 frequency histogram of only supraorbital crest landmarks. **C)** Wire frame graph of only eye and nape landmarks (#1, 2, 7, 8, 9, 10) with corresponding CV1 frequency histogram. Males = light blue; Females = red.

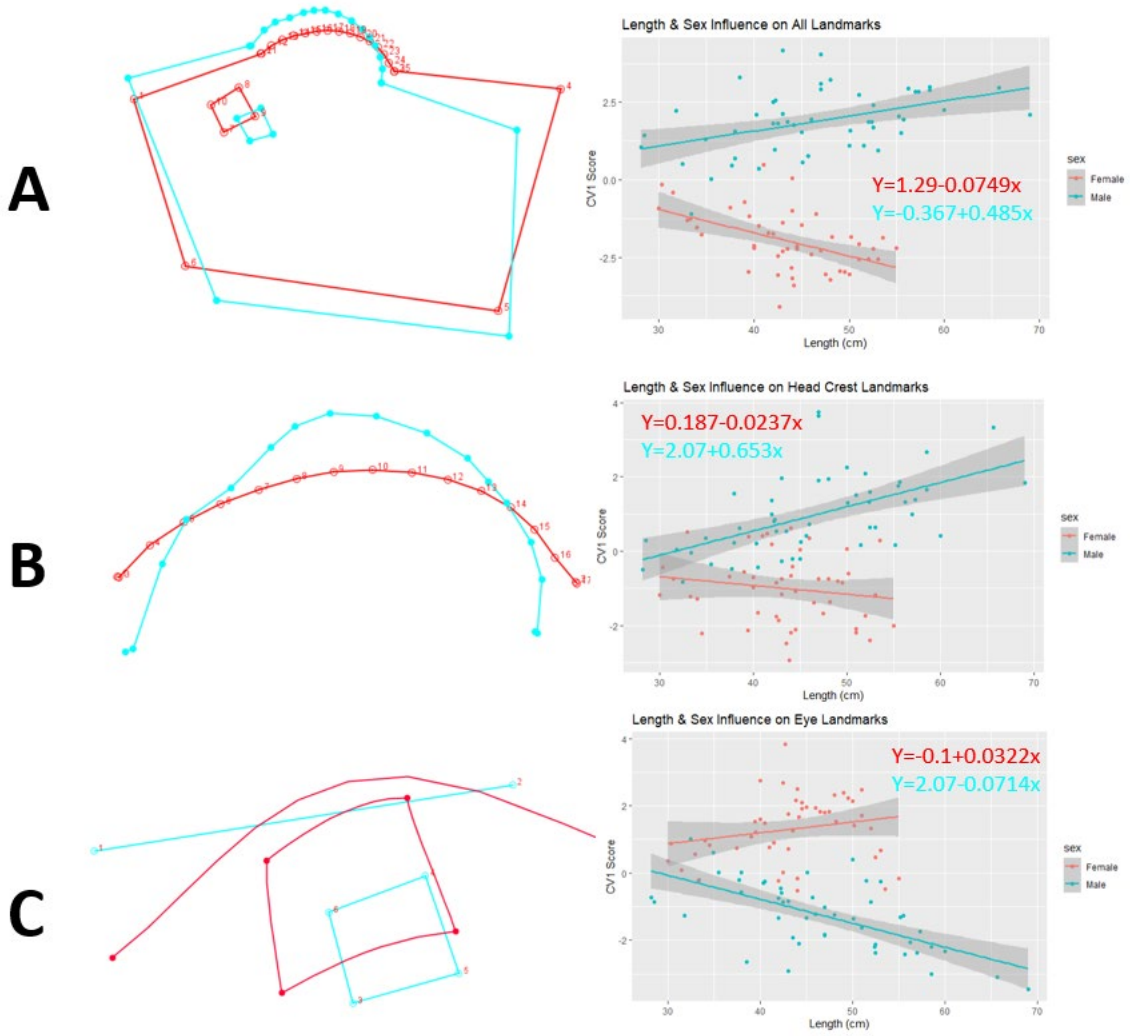

Figure 5. Shape change of different structures in relation to fish length A) Wire frame of all landmarks (left). Linear regression of CV1 in relation to fish length. (right). **B)** Wire frame of only supraorbital crest landmarks (right) with corresponding regression of CV1 in relation to fish length (right). **C)** Wire frame of only eye and nape landmarks (left) with corresponding regression of CV1 in relation to fish length. Males = light blue; Females = red

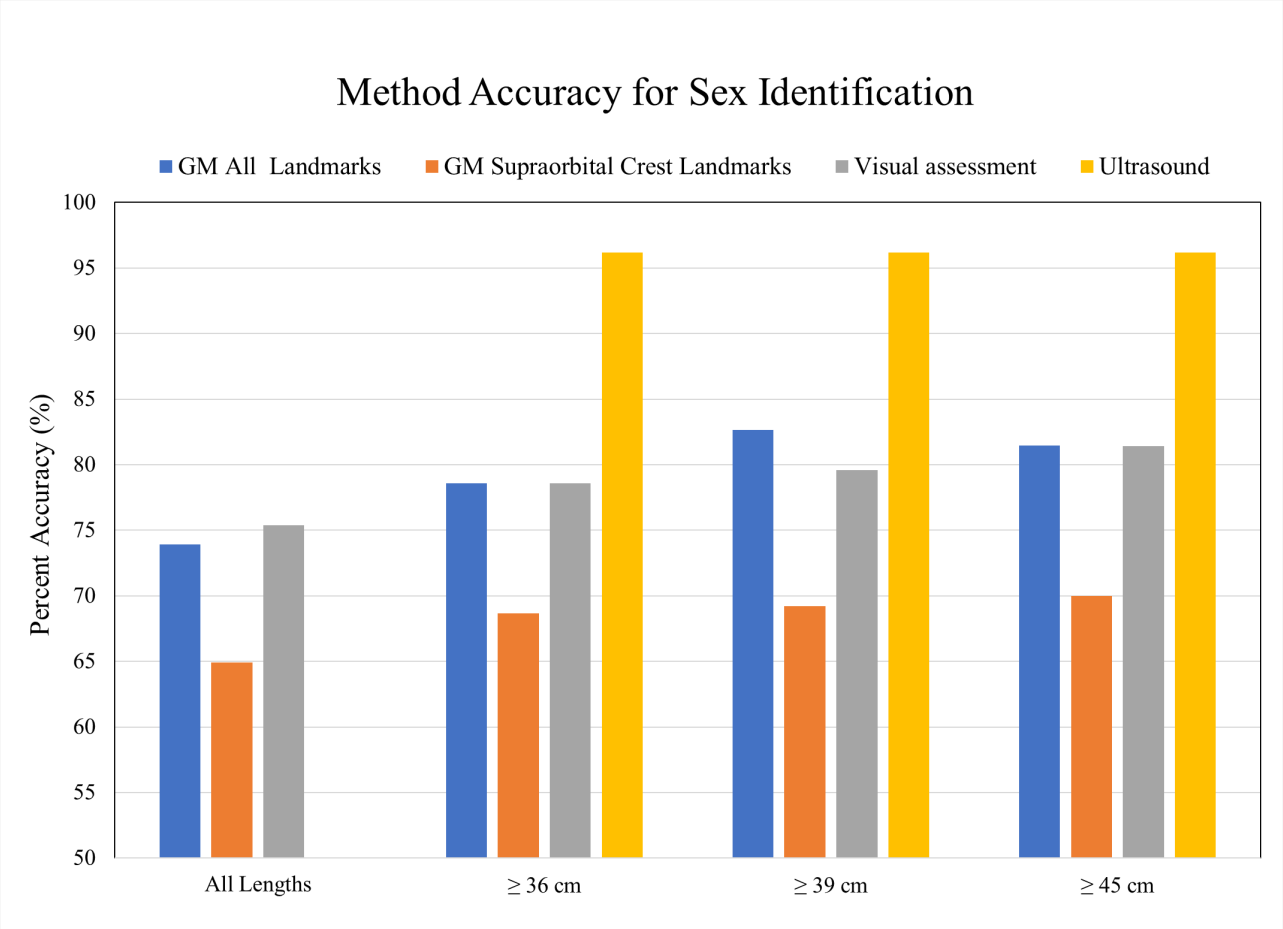

Figure 6. Method accuracy for sex identification. Percent accuracy for each method of sex identification at different lengths using different sets of landmarks with geometric morphometrics (GM), ultrasound, and visual assessment. Sex was later confirmed by dissection and visual inspection of the gonads.

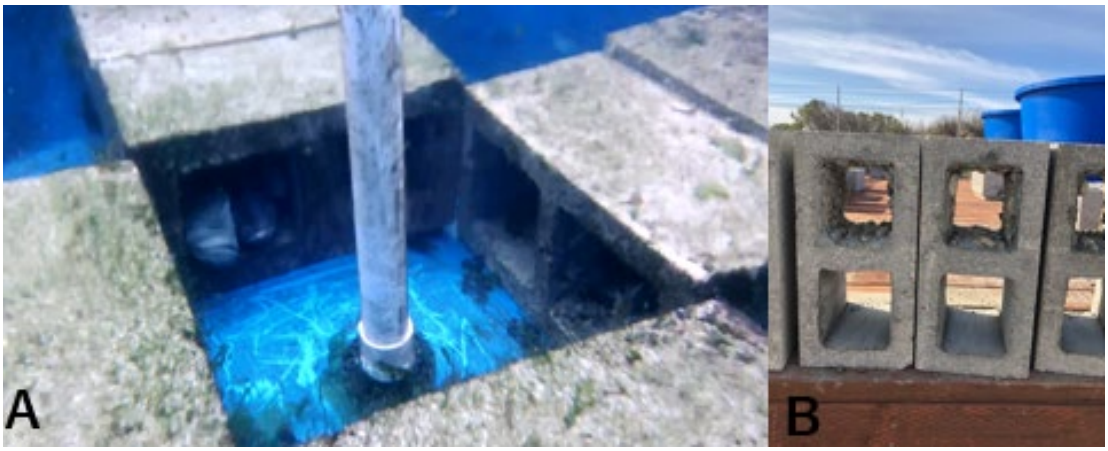

Figure 7. Design of experimental habitat. A) Tank habitat consisted of constructed rocky cave dwellings by arranging cinder blocks inside the tanks to mimic natural rock caves. The arrangement of 8 caves per tank provided each male and female the choice of either a rough or smooth cave. **B)** cinderblocks depicting rough (top) and smooth (bottom) spawning substrates.

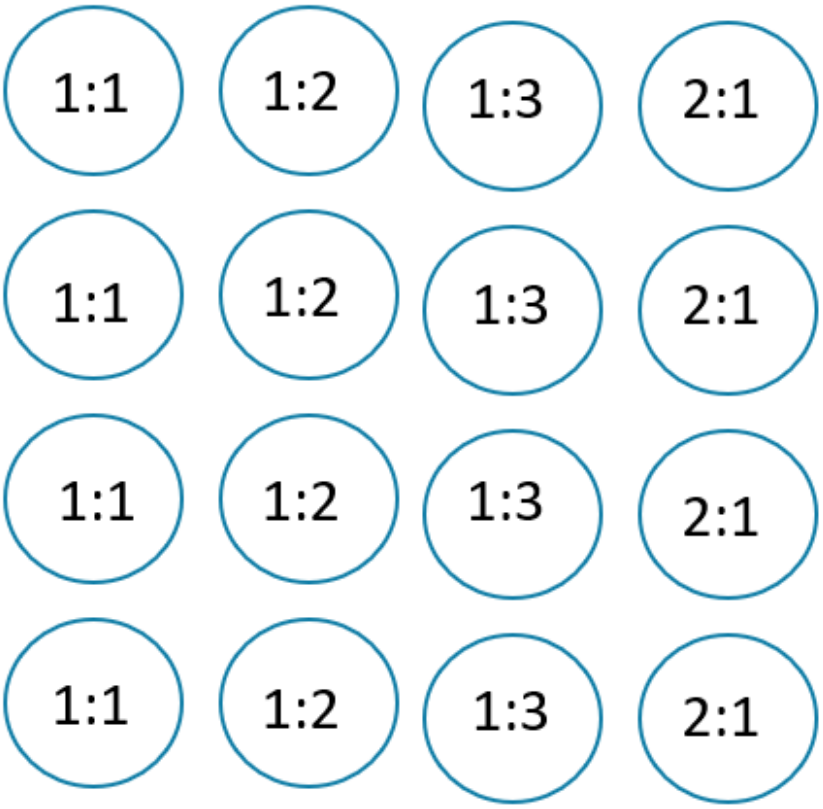

Figure 8. Experimental design for reproduction experiment #2. Experimental arrangement of tanks and sex ratio (male : female) treatments.

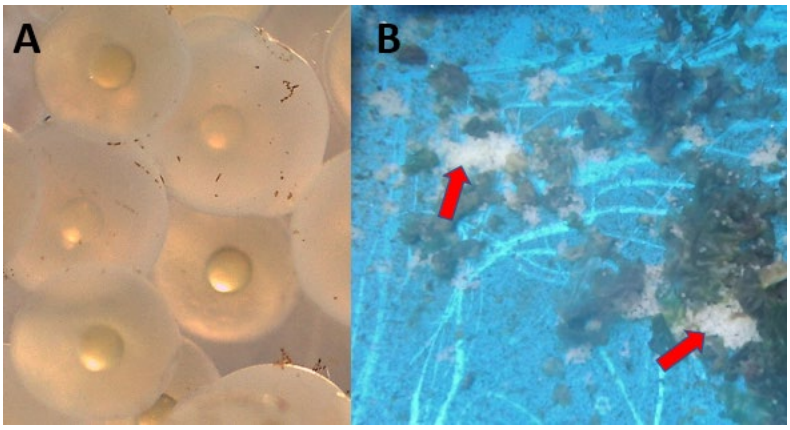

Figure 9. Photos of unfertilized eggs in reproduction experiment #1. A) 4.2x zoom on a cluster of eggs released from a female. **B)** Unfertilized eggs dispersed around the reproduction tank. This photo displays many unfertilized eggs (white spheres) that were released from one of the females in the tank. This is one of the 15 unsuccessful spawning events.

Figure 10. Male parental care of fertilized egg masses in reproduction experiment #2. A) Parental care was observed from the video of fanning behavior using its caudal fin and tail wrapped around the eggs. **B)** Egg masses were light-yellow in color, small, and not adhesive to substrate, but adhesive to other eggs within the mass.

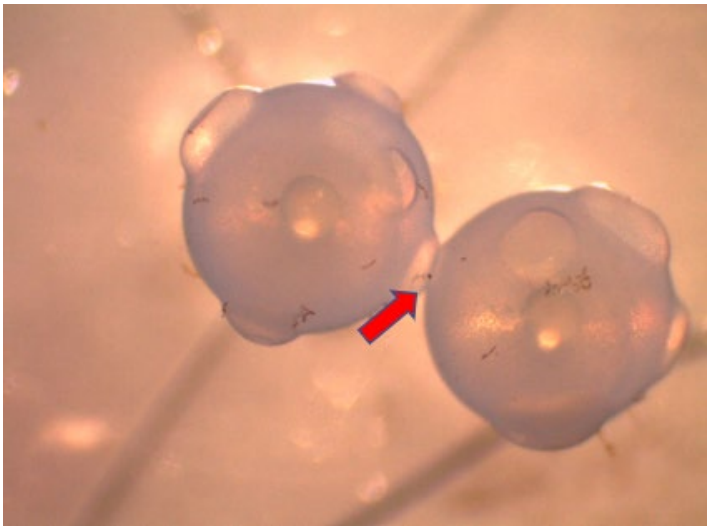

Figure 11. Fertilized eggs from reproduction experiment #1. 4.2x zoom on a cluster of fertilized eggs. Note the adhesive pad ornamentation on the outside of the opaque chorion.

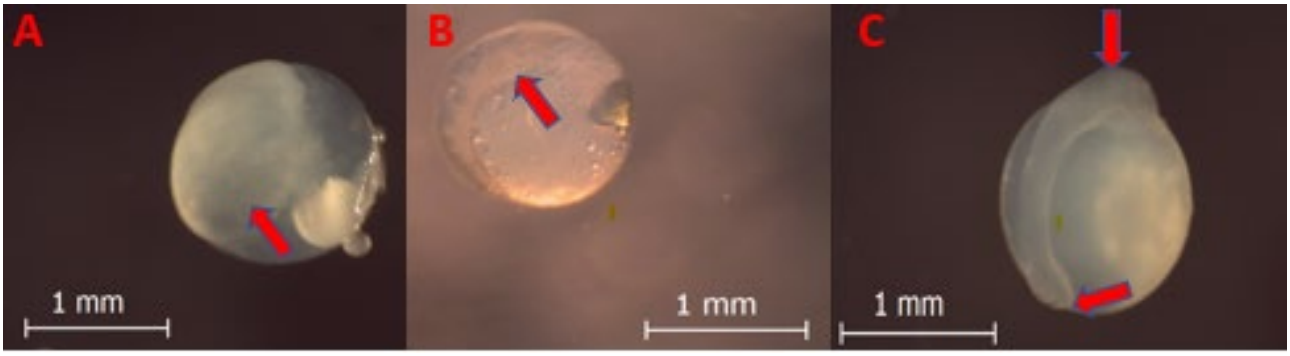

Figure 12. Gastrula stage of developing eggs in reproduction experiment #2. A) Arrow points to the animal pole of cells that denote the 50% epiboly stage (1 DPF). **B)** Arrow shows the widened area denoting the embryonic shield (2 DPF). **C)** Bottom arrow displays the development of the tail bud, while the top arrow denotes the first developments of the head (3 DPF).

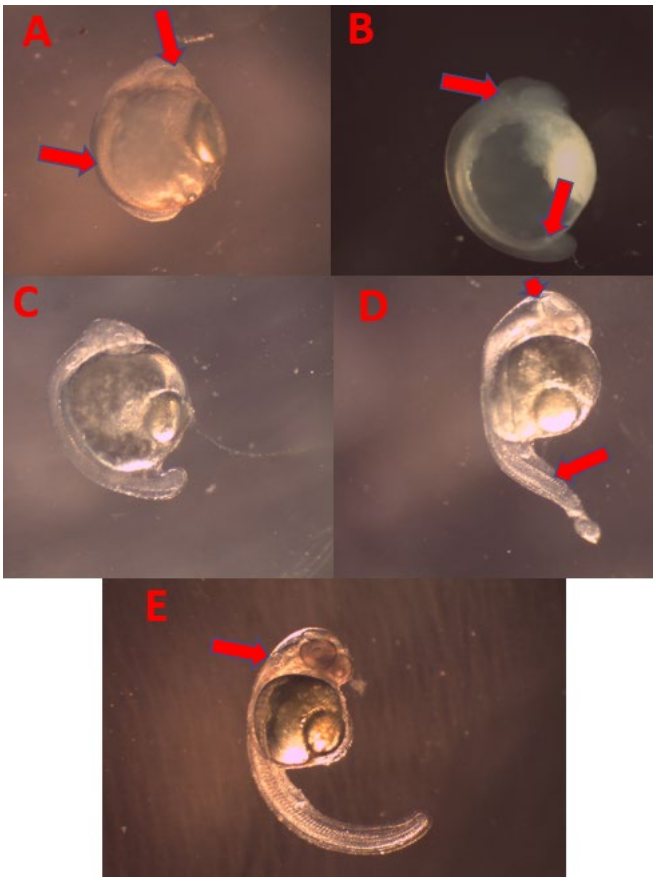

Figure 13. Segmentation period of developing embryos in reproduction experiment #2. A) DPF 5 - the first 8 somites and the first development of the optic vesicle (upper arrow). **B**) DPF 6 - marks the first observation of the hindbrain development (upper arrow) and the beginning development of the Kupfer's vesicle (lower arrow). **C)** DPF 7 - shows further development of the optic vesicle, Kupfer's vesicle, and the beginning of tail development. **D)** DPF 8 - the tail becomes well extended from the yolk (upper arrow) and there are observable divisions of the hindbrain where there is a hollowed-out area of the otic vesicle. Stack of pennies appearance along the trunk of the embryo denotes somite development (lower arrow). **E)** DPF 9 - more muscle development along the notochord was observed along with spontaneous myotomal contractions produced the first twitching and lashing from side to side. At this stage the tail is well extended. The first observation of the otoliths (arrow).

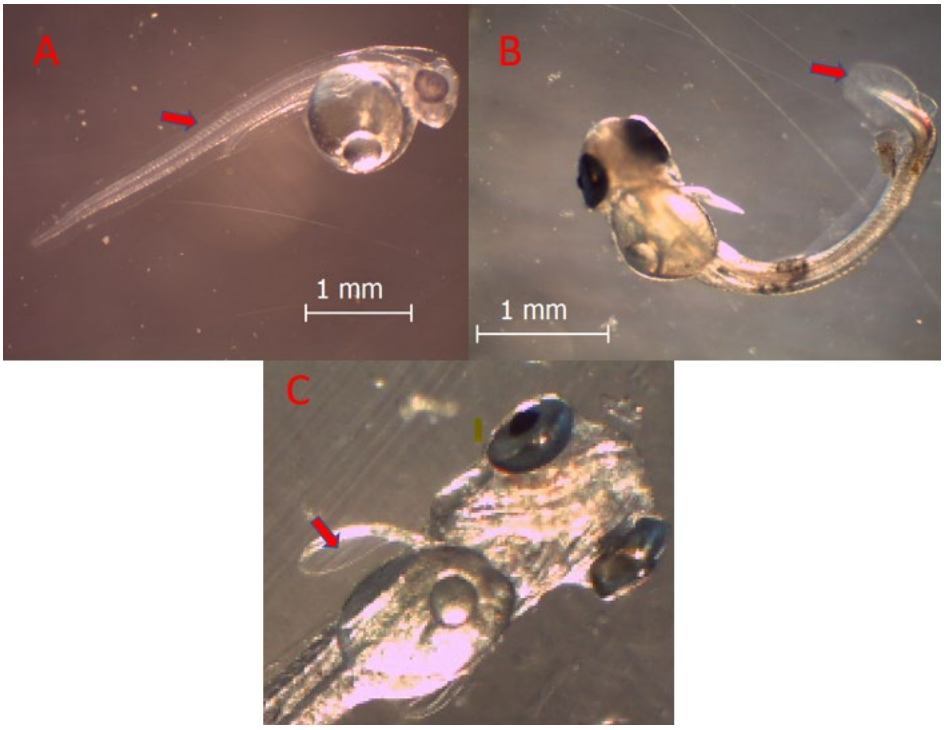

Figure 14. Fin development of developing embryos in reproduction experiment #2. A) lateral view of larval showing dorsal fin development at 10 DPF. **B)** ventral view of larva showing caudal tail development near the end of incubation. (21 DPF). **C)** ventral view of larva showing pectoral fin development (21 DPF).

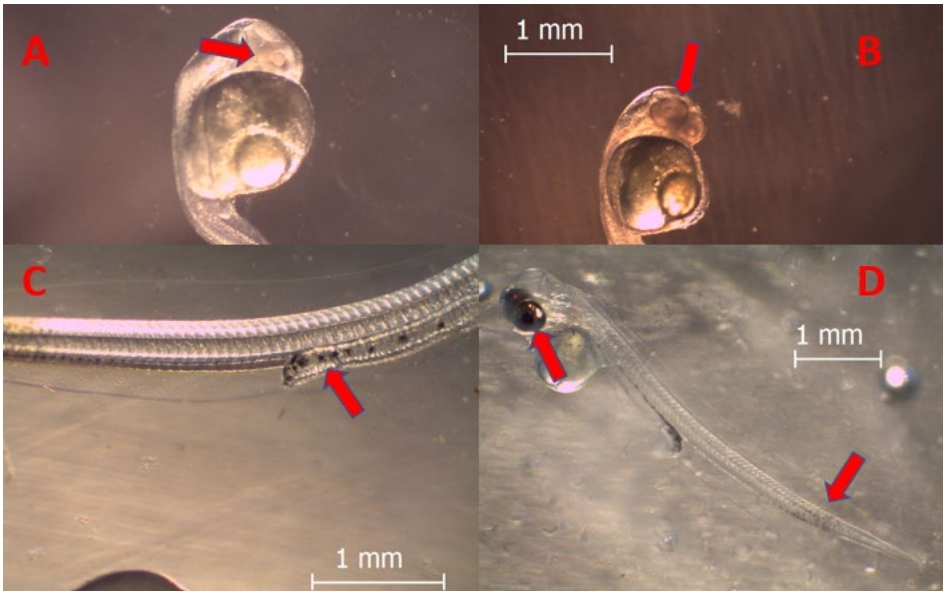

Figure 15. Eye and body pigmentation during embryological development in reproduction experiment #2. A) DPF 8 before any pigmentation starts. **B)** DPF 9 beginning of pigmentation starting with retinal epithelium. **C)** intestinal pigmentation (21 DPF). **D)** Completed eye pigmentation (upper arrow). Dotted melanophores on postanal region and a wide speckled band near caudal peduncle (lower arrow) (23 DPF).

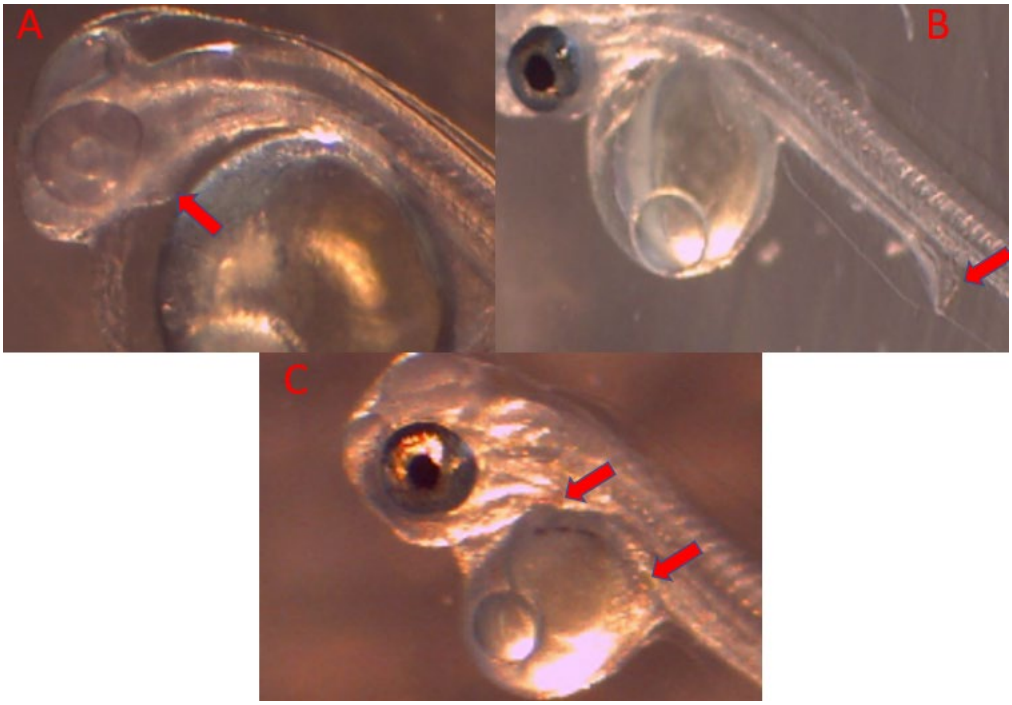

Figure 16. Circulatory system development of embryos in reproduction experiment #2. A) first observation of beating cone shaped heart (9 DPF). **B**) First observation of "blood island" (14 DPF). **C**) Observations of blood around yolk sack near the heart (18 DPF).

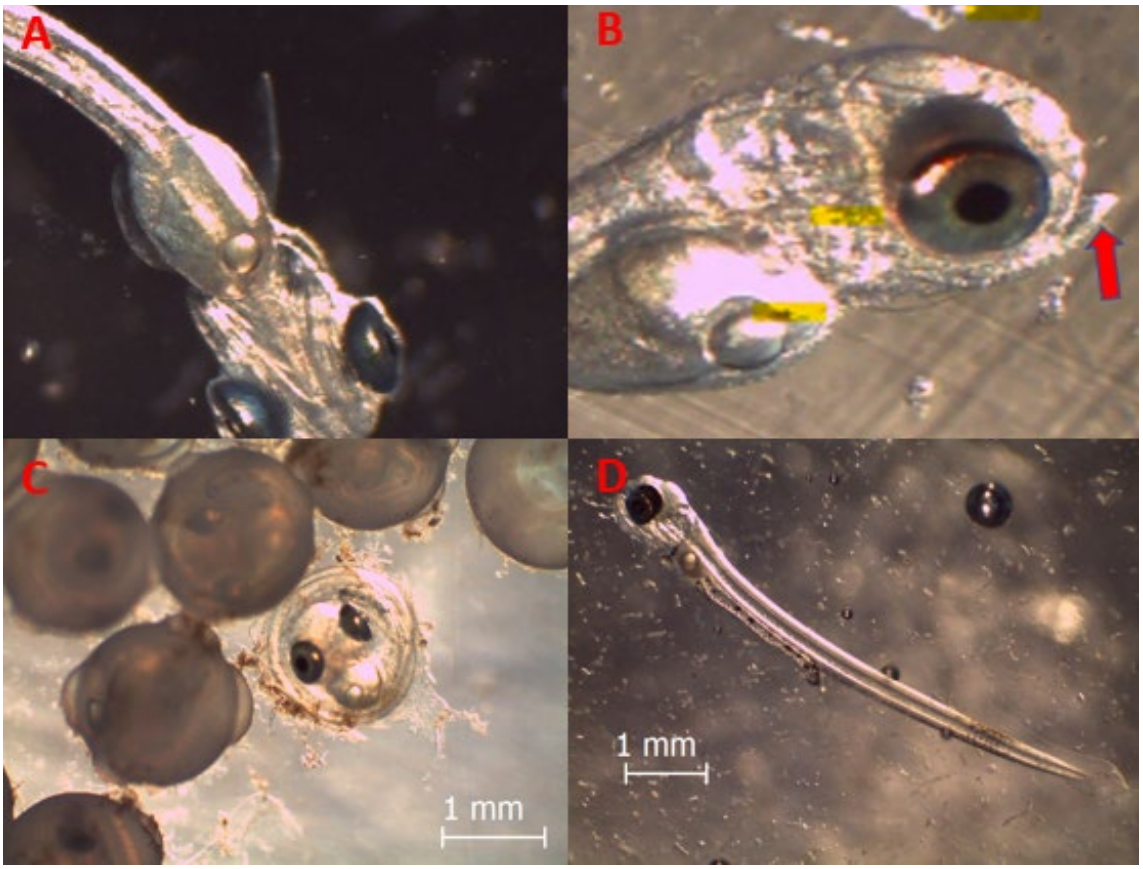

Figure 17. Later stages of development in reproduction experiment #2. A) Pectoral fin development. Zoomed in ventral view of embryo with a fully developed pectoral fin. **B)** Mouth development. Embryo with a fully developed mouth before hatching. **C)** Hatching period**.** Opaque eggs are enveloped with a chorion. The egg's transparent chorion degrades right before hatching, leaving only a thin membrane for the larvae to hatch through. **D)** Photo of newly hatched larva (average size = 7.43 mm).

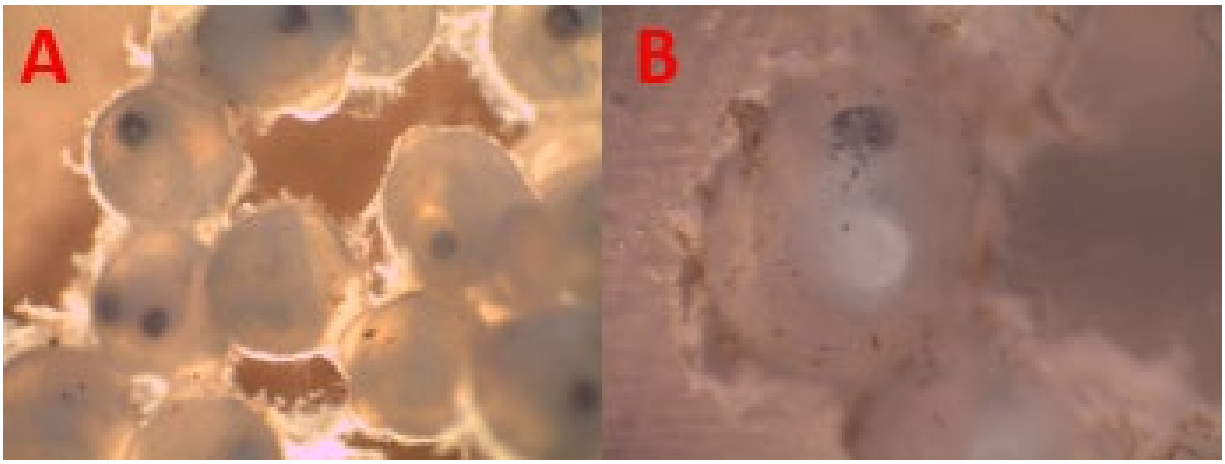

Figure 18. Eggs infected with unidentified fungus. A) Depicts a photo of part of the egg that has been infected by and unidentified fungus. **B)** Displays a zoomed-in version the dead embryo (4.2X) that is decomposing within the egg, with mortality likely caused by the infection. The fungus is white and filamentous, adhering to the outside of the chorion. Unfortunately, most of the egg mass became infected by the same fungus.

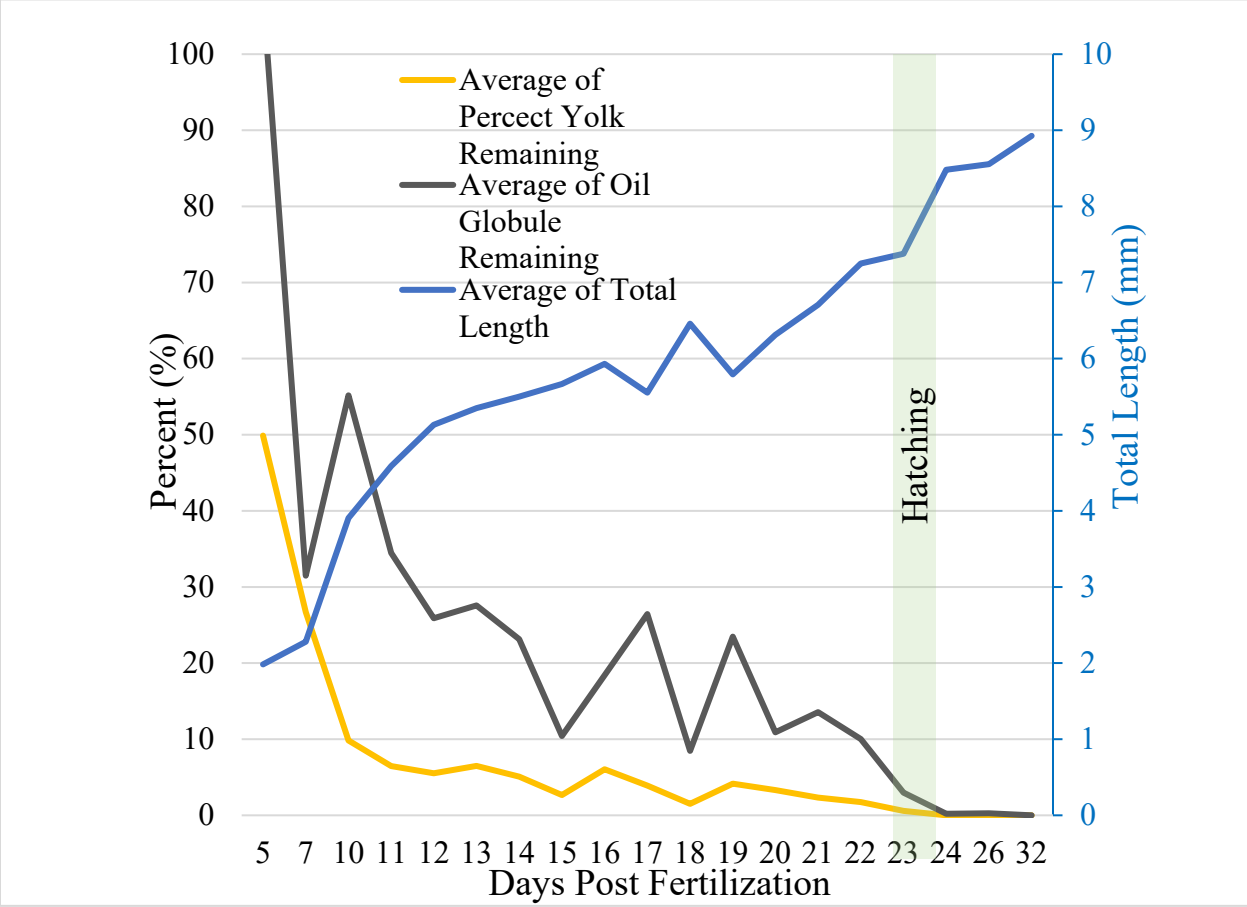

Figure 19. Average yolk and oil globule depletion and embryological and larval growth chart. 3-5 embryos and larvae were sampled daily document endogenous resource use during incubation. This chart displays total length (mm) (blue line), the percent of yolk depletion (yellow line) and the percent of oil globule depletion (black line) on the y-axis with respect to days post fertilization and hatching. The majority of the yolk is depleted by day 10, while the oil globule volume remains above 10% until hatching and only becomes depleted 2 days post hatching.

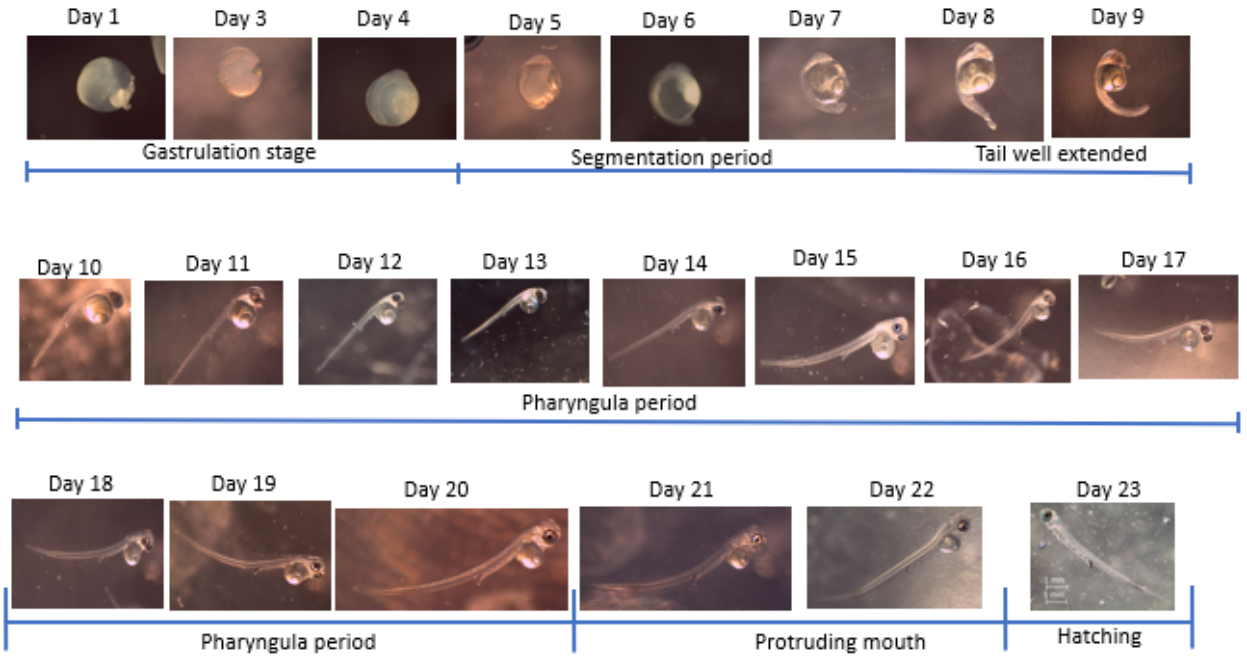

Figure 20. Embryological timeline. Days post fertilization displayed in sequence separated by embryological stage.

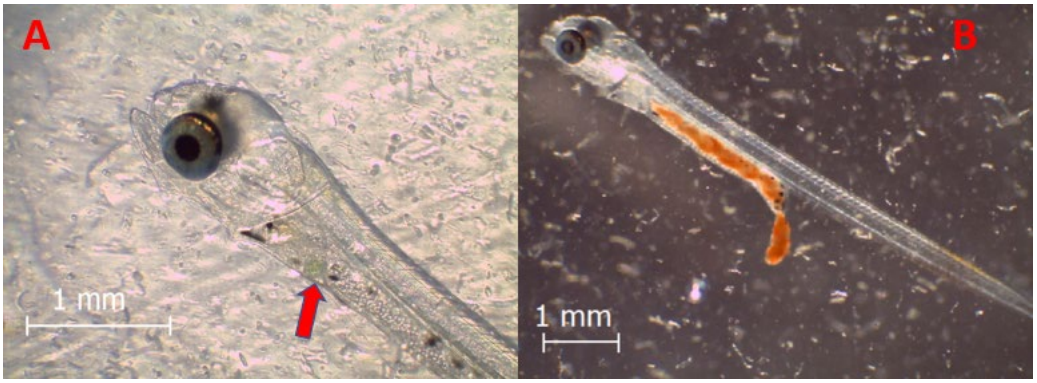

Figure 21. Larval period and first feeding. A) Depiction of first rotifer feeding as seen by green rotifers inside larval stomach. **B)** First feeding of *Artemia* sp. as seen from bright orange *Artemia* within the larva's intestine, which are actively being excreted.