Improving Conservation Hatchery Effectiveness: A Study on Alternative Egg Treatments for Delta Smelt *Hypomesus transpacificus* and Longfin Smelt *Spirinchus thaleichthys*

By

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Abstract

At the UC Davis Fish Conservation and Culture Laboratory (FCCL), Delta Smelt (*Hypomesus transpacificus*) and Longfin Smelt (*Spirinchus thaleichthys*) are being cultured for conservation. While the aquaculture processes for these species were established, they are not perfect and demand extensive labor and time, particularly for fish egg treatments. This study explored alternative egg separation and adhesiveness removal methods. In the first part of the study, Epsom salt solutions with varying specific gravity (SG) were utilized to separate live and dead eggs. The results showed promising effectiveness for specific gravity value of 1.08, 1.10, and 1.12, which effectively separated dead eggs for both species with minimal impacts on hatching rates. In the second part of the study, Ramshorn Snails (*Helisoma anceps*) were utilized as natural egg separators. The result showed these snails preferred dead Delta Smelt eggs compared to live eggs. This behavior was first discovered and opened a potential route for future fish egg separation methods. In the last part of the study, alternative fish egg adhesiveness removal methods were compared to the traditional bentonite clay method. The results showed excellent egg adhesiveness removal capability for sodium hypochlorite and tannic acid, while the effectiveness of Alcalase enzyme increased with concentration and achieve the same effectiveness at the higher concentration of 5% (v/v). However, the results also showed the negative impacts of sodium hypochlorite to the hatching rate of Delta Smelt eggs, which no eggs hatched after being treated. Tannic acid and Alcalase, on the other hand, showed no significant impact to the hatching rate. From the findings of this study, the Epsom salt solution method for fish egg separation has been successfully integrated to the hatchery-scale fish production at the FCCL, yielding notable time and labor savings for egg treatments. However, the approaches using Ramshorn Snails and alternative adhesiveness removal techniques require further study and optimization.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Delta Smelt (*Hypomesus transpacificus*) and Longfin Smelt (*Spirinchus thaleichthys*) are small fish species endemic to the upper Sacramento-San Joaquin estuary in California (McAllister, 1963; Moyle, 1976; Wang, 1986). Unfortunately, the populations of both Delta Smelt and Longfin Smelt have experienced significant declines in abundance in recent years. Their ability to persist in the estuary is now in doubt due to major environmental changes, including increased diversion of freshwater inflow for irrigated agriculture and urban uses (Moyle et al., 1989; Nichols et al., 1986; Williams et al., 1989). To avoid the potential extinction of Delta Smelt, a refuge population has been established to safeguard the evolutionary potential of a species for many generations within a controlled, captive refuge setting at the UC Davis Fish Conservation and Culture Laboratory (FCCL), a university-operated conservation hatchery, since 2008 (Ellison et al., 2023; Lindberg et al., 2013). A similar refuge population for Longfin Smelt is also in the planning stages now. In order to protect them from extinction and to construct healthy refuge populations, culture techniques for these fish have thus far focused on maximizing genetic diversity through large-scale operations (Tsai et al., 2022b).

The biology and reproductive characteristics of these species play a critical role in their conservation and management. Delta Smelt are euryhaline fish, capable of tolerating a wide range of salinities. During the late winter and early spring months, typically from December through March, Delta Smelt migrate upstream to the freshwater regions of the upper estuary for spawning (Feyrer et al., 2011). Simultaneously, Longfin Smelt take a similar journey during the winter months (December to February), migrate upstream towards the confluence of the Sacramento and San Joaquin Rivers for their spawning

season. It is hypothesized that Longfin Smelt employ a benthic spawning strategy, fastening their adhesive eggs to sandy substrates within the freshwater environment (Moulton, 1974; Moyle, 2002; Rosenfield & Baxter, 2007). The spawning behavior of both species involves external fertilization, with females releasing adhesive eggs that stick to substrates in their environment (Bennett, 2005; Feyrer et al., 2011; Tsai et al., 2021). Every year at the FCCL, more than 500,000 eggs are spawned. During the process, the adhesiveness of the fish eggs is removed by using bentonite clay (Sigma-Aldrich, Inc.,Saint Louis, Missouri, USA; Lindberg et al., 2013; Tsai et al., 2022b). After the removal of the adhesive nests, one crucial step in the culture process is to separate fertilized eggs from unfertilized ones for each single-pair cross (about 1,500 eggs per cross for 1-year-old fish; Lindberg et al., 2013; Tsai et al., 2022b). Dead or unfertilized eggs are susceptible to fungal infections. For instance, unfertilized salmon and trout eggs can be infected by *Saprolegnia* (Leitritz & Lewis, 1980), which can spread to fertilized eggs, leading to a high mortality rate (Figure 1.1). The current dead egg removal method implemented at the FCCL is to manually separate them by using polyethylene plastic pipettes (Fisherbrand™ Standard Disposable Transfer Pipettes, capacity: 7.7 mL; Molecular Bio Products Inc., San Diego, California, USA). For the refuge population, 700 fertilized fish eggs are manually picked up from each single-pair cross and consolidated with eggs from another 7 crosses for incubation (Tsai et al., 2022b). However, this meticulous process of egg separation is particularly labor-intensive and time-consuming.

The egg de-adhesive treatments and separation processes are critical to the successful hatching of the eggs, the production of larvae, and the maintenance of a genetically managed population. It is essential for the long-term health and adaptability to the wild environment of the species. The ultimate goal is to release these genetically diverse individuals back into the wild, where they can contribute to the recruitment and recovery

of wild populations and help re-establish a balanced ecosystem in the Sacramento-San Joaquin estuary. Efficient egg separation and treatment methods are therefore essential for the success of these programs.

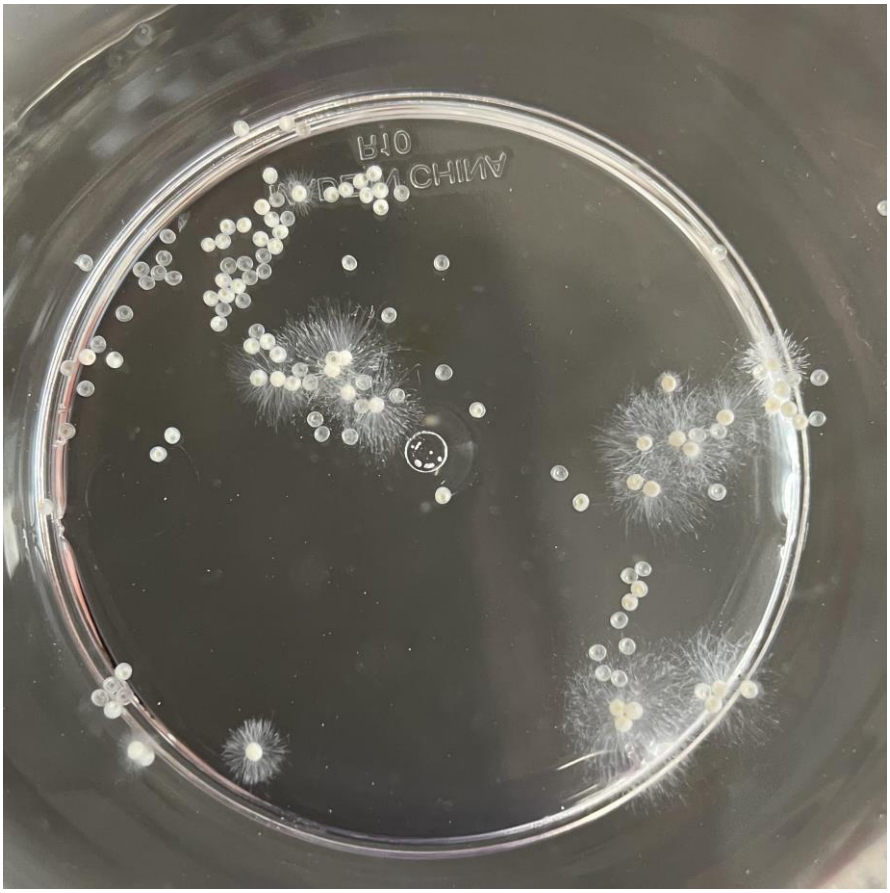

Figure 1.1 Fungal growth on Delta Smelt eggs (photo taken on 3 days post fertilization). The fungus proliferates on dead and unfertilized eggs, posing a threat to live eggs by potentially spreading out on them and causing their death.

1.2 Adhesiveness of Fish Eggs Across Species: From Biological Adaptation to Aquaculture Challenges

Fish eggs exhibit diverse adhesive characteristics depending on the species and environment, ranging from non-adhesive pelagic eggs to adhesive demersal eggs that attach to substrates (Siddique et al., 2016). This adhesiveness is a critical biological trait, facilitating survival and development of the embryos in varying ecological conditions. Most fish oocytes and eggs are enveloped by a thick proteinaceous layer, commonly

referred to as the egg envelope or chorion (Cherr & Clark Jr, 1982). The structure of the chorion exhibits adaptations to differing environmental conditions (Rizzo et al., 2002). For example, pelagic eggs typically feature a thin follicular epithelium layer and zona radiata, while demersal eggs are encompassed by a thicker follicular epithelium layer and a more intricate egg envelope (Stehr & Hawkes, 1979). The egg envelope serves a multitude of protective functions. It shields the egg from mechanical forces such as water currents and predators (Paxton & Willoughby, 2000; Riehl & Patzner, 1998; Rizzo et al., 2002; Żelazowska, 2010). Additionally, the envelope plays a role as a diffusion barrier against environmental and chemical pollutants (Esmaeili & Johal, 2005).

Adhesiveness in fish eggs is primarily achieved through a sticky chorionic layer surrounding the egg, which enables the egg to adhere to various substrates in the aquatic environment (Riehl & Patzner, 1998; Yanagimachi et al., 1992). The adhesive nature of fish eggs varies greatly among species. While some species lay eggs that are non-adhesive and remain in the water column (pelagic eggs), others lay adhesive eggs (demersal eggs) that stick to substrates (Riehl & Patzner, 1998; Siddique et al., 2016). This adhesive trait is an evolutionary adaptation to increase egg survival, helping to prevent eggs from being swept away by currents or consumed by predators. The adhesiveness of fish eggs can be affected by several environmental factors, such as salinity (Mansour et al., 2009). This suggests that the adhesiveness of fish eggs may be affected by environmental conditions, including changes in salinity that are prevalent in the estuarine habitats of Delta Smelt and Longfin Smelt.

In order to perform effective aquaculture of fish, it is required to obtain knowledge of each step of the life cycle for different species (Geist, 2011; Pander & Geist, 2013). In this thesis, the study focuses on the egg stage of Delta Smelt and Longfin Smelt. Several experiments have been conducted on the adhesiveness characteristics of the fish eggs.

Nagel et al. (2021) used scanning electron microscopy to assess the egg surface quality of Common Nase (*Chondrostoma nasus* L.) and found significant differences in egg surface properties among individual females and among different populations, mainly attributed to body length variability and merging of adhesive villi as well as to coating and filament-like connections of these structures. The study highlights the need for further investigations to better understand the relationship of egg surface properties, egg adhesiveness, and hatching success to understand the recruitment ecology and performance of early life stages in freshwater fish. Another study investigated the effects of diameter, density, and adhesiveness on the settling velocity and drag coefficient of fertilized eggs from the Siberian Sturgeon (*Acipenserbaeri Brandt*) and Amur Sturgeon (*Acipenser schrenckii*) in water flow and found the adhesiveness decreases the terminal settling velocity (Yi et al., 2022). The study provides an extensive analysis of the adhesiveness characteristic and its impact on egg movement in the water.

Research shows that the causes and types of egg adhesiveness vary significantly across diverse species. The adhesive properties of the eggs from different species serve crucial functions, which act as a defense mechanism, preventing egg dispersal caused by water currents, waves, or predation (Feyrer et al., 2011). Delta Smelt and Longfin Smelt also lay adhesive eggs (Bennett, 2005; Lindberg et al., 2020; Moyle, 2002). Various theories have been proposed about the preferred spawning habitats of Delta Smelt. These theories suggest the adhesive eggs may be deposited on sandy beaches (Bennett, 2005; Moyle et al., 2016), within submerged tree branches (Wang, 1986), amongst larger rock rubble (Bennett, 2005), or in open water situated above sandy or rocky substrates (Wang, 1986). Additionally, studies suggested that Delta Smelt tend to lay more eggs under conditions of higher water flow velocity (Lindberg et al., 2020). This typically occurs during forward-swimming dashes while the fish are in direct contact with the substrate

(Tsai et al., 2022a). However, while egg adhesiveness confers advantages in the wild, it poses challenges in a hatchery setting. These challenges can include fungal infection, as well as difficulties in separating fertilized from unfertilized eggs (Leitritz & Lewis, 1980). Despite the mechanisms behind the adhesiveness of Delta Smelt and Longfin Smelt eggs not being fully understood, the application of bentonite clay has been demonstrated to effectively reduce this adhesiveness (Lindberg et al., 2013). Nevertheless, there might be better alternatives as bentonite clay often fails to entirely eliminate the adhesiveness, leading to instances where eggs stick to each other during the separation process, thus posing a challenge to successful separation and incubation. Understanding these natural adaptive mechanisms and their implications in human-controlled environments is the key for the development of effective conservation practices for Delta Smelt and Longfin Smelt.

1.3 Density of Fish Eggs: How They Shape Survival and Conservation Strategies

Another important physical property of fish eggs is their density. Chen et al. (2021) examined the evolution of alternative floating mechanisms in freshwater fish eggs and found that while the buoyancy of freshwater fish eggs has been suggested to be determined primarily through the presence of oil droplets, the majority of freshwater pelagic eggs do not possess an oil droplet (Figure 1.2; Carter et al., [2015]). The study applied a physical model of buoyancy to understand the contributions of oil droplets and hydration to the buoyancy of pelagic freshwater fish eggs and found that the probability of possessing oil droplets in freshwater pelagic eggs exhibits a significant negative relationship with the size of perivitelline space (Figure 1.3; Romney et al., [2019]), which may reflect a tradeoff relating to energy allocation in contrasting habitats. Another study assessed the variability in egg specific gravity for selected local populations of Atlantic Cod (*Gadus morhua*) and found that the specific gravity of eggs is positively correlated with egg survival and female

spawner age and size (Jung et al., 2012). The study not only established a field-based correlation between egg diameter and buoyancy, or floating depth, but also significant variations in the horizontal extent of spawning habitat and the impact of oxygen levels on egg survival. These findings underscore the importance of incorporating environmental information into ecosystem-based management strategies. In another study, theoretical model was used in combination with empirical analysis to investigate the transport and dispersion of the Asian carp (Black Carp [*Mylopharyngodon piceus*], Grass Carp [*Ctenopharyngodon idellus*], Silver Carp [*Hypophthalmichthys molitrix*], and Bighead Carp [*Aristichthys nobilis*]) eggs in the middle Yangtze River by Reynolds-averaged numerical simulation and found that fish eggs of four major Asian carp were generalized to sediment-like particles (Yang et al., 2019). The study illustrated a good view of the density property in fish eggs and showed an analytic solution considering the change of lateral velocity along the curved channels in rivers and used an improved three-dimensional hydrodynamic model to simulate the distribution characteristics of fish eggs, treating them as sediment-like particles within the curved channel. Finally, a study examined the effective spawning stock biomass of an exploited fish species in the Baltic Sea and found that salinity affects neutral egg buoyancy, which is positively correlated with egg survival, as only water columns away from the oxygen consumption-dominated sea bottom contain sufficient oxygen. The study established a field-based relationship between egg diameter and buoyancy (floating depth) and quantified the number of effective spawners, which are able to reproduce under ambient hydrographic conditions (Hinrichsen et al., 2016). It also highlighted the importance of including environmental information such as water salinity and oxygen concentration in ecosystem-based management approaches.

Collectively, these studies offer significant insights into the characteristics of density and buoyancy of fish eggs and the complex interplay of factors that influence their

survival and reproduction. Understanding these physical properties and ecological interactions is not just academically intriguing, but also of practical importance. Specifically, such knowledge can illustrate the design and optimization of novel fish egg separation and treatment techniques. For Delta Smelt and Longfin Smelt, these advancements could potentially improve egg handling procedures, enhance hatchery operations, and ultimately contribute to more effective conservation strategies.

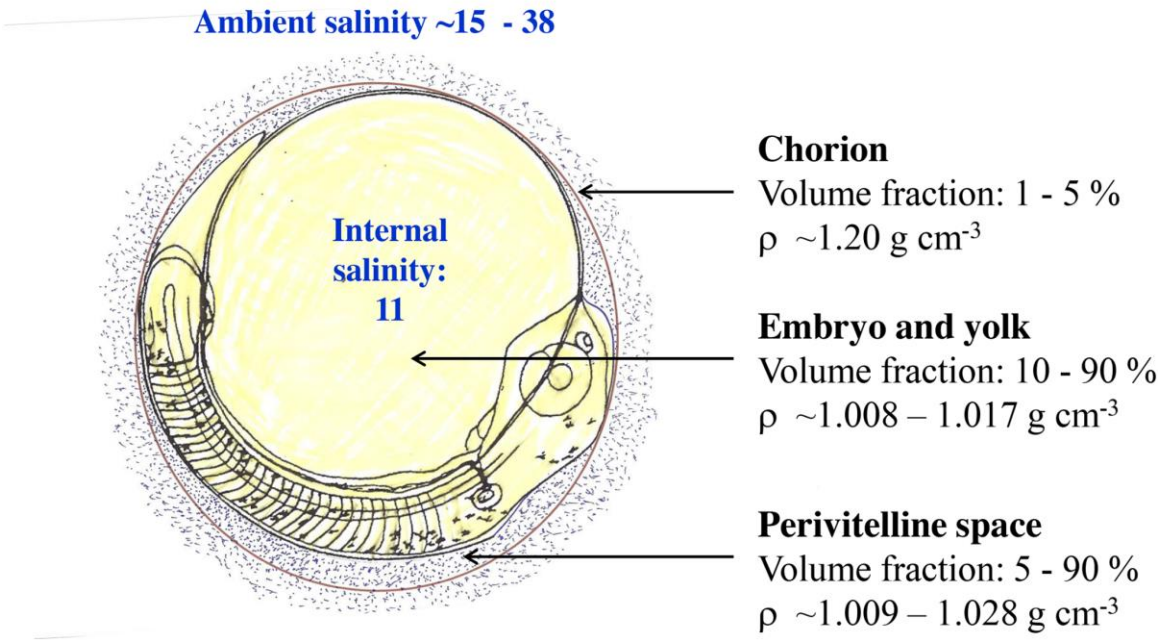

Figure 1.2 The physical-biological attributes of teleost eggs comprise several components (ρ = density). The total specific gravity of the egg is determined by the fractional contributions of three key elements: 1) the chorion, 2) the embryo and yolk, and 3) the perivitelline space (Sundby & Kristiansen, 2015).

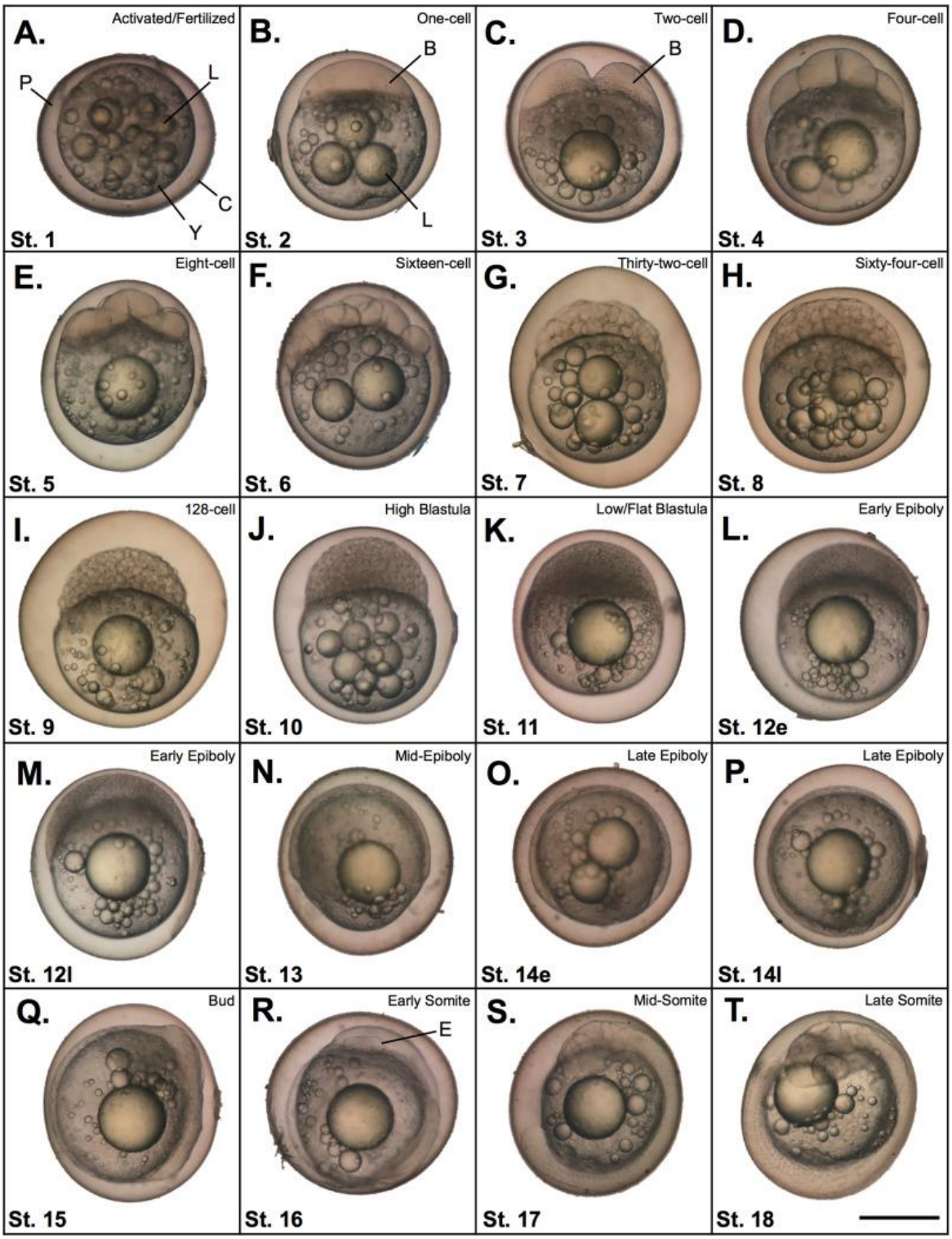

Figure 1.3 Morphological stages of embryonic development in Delta Smelt, from fertilization through somitogenesis. Each panel, labeled from A to T, displays a different stage. The stage number (St.) is positioned at the bottom left of each panel, while the stage name is found at the top right. In these depictions, 'C' = chorion, 'P' = perivitelline space, 'Y' = yolk, 'L' = lipid droplet (oil droplet), 'B' = blastomere, and 'E' = eye. A scale bar situated at the bottom right of the figure represents 500 μm (Romney et al., 2019).

1.4 Current Methods for Fish Eggs Separation: Limitations and Challenges

Several techniques have been employed to separate unfertilized salmonid eggs from eyed eggs (Leitritz & Lewis, 1980; Weithman & Anderson, 1977). Similar to Delta Smelt and Longfin Smelt, for the cultivation of trout (*Oncorhynchus spp.*) and salmon (*Salmo spp.*), the egg separation process is necessary unless a fungicide is used to prevent dead eggs from fungal growth and the fungus spreading to the fertile ones. This step is called egg picking (Leitritz & Lewis, 1980). Various methods include the traditional hand picking method, the pipette method, the salt flotation method, and mechanical egg sorters have been applied to achieve such a goal (Leitritz & Lewis, 1980; McMullen, 1948; Smith, 1950).

The hand picking method, traditionally used in trout and salmon farms, employs large metal tweezers as the primary tool for this task (Leitritz & Lewis, 1980). However, this method is extremely slow. The advent of the pipette, universally accepted as a tool for egg picking, significantly improved this process. The pipette, essentially a long tube attached to a small syringe bulb, should have an internal diameter large enough to fit the eggs. Skilled workers can sort eggs relatively quickly with this tool, but the process can still be physically demanding and potentially lead to severe eye fatigue (Leitritz & Lewis, 1980). In California, pipettes are primarily used to sort small quantities of trout and salmon eggs before they are transported (Leitritz & Lewis, 1980). The pipette method has been employed for many species (Leitritz & Lewis, 1980; Rohani et al., 2019), including Delta Smelt and Longfin Smelt at the FCCL. Despite its wide usage, this method remains labor-intensive and time-consuming.

Other egg separation methods, such as mechanical egg sorters and the salt flotation methods developed for larger fish species like salmon and trout, often rely on size, color, and density differences to separate fertilized and unfertilized eggs (Agulleiro et al., 2006; Ciereszko et al., 2006; Leitritz & Lewis, 1980). The Power Egg Picker (Figure 1.4), an

enhanced version of the pipette method developed by McMullen in 1948, was designed to supersede the traditional method of hand-picking eggs with pipettes or tweezers. While the device enhances the efficiency of egg separation, it still requires human intervention to determine the viability of the eggs. Another method for the egg separation is the salt flotation method, involving the use of a salt box large enough to accommodate a standard egg basket (Figure 1.5). The box is nearly filled with water, into which common stock or table salt is mixed until an appropriate concentration is achieved. The optimal amount of salt can be determined experimentally by sampling the solution in a container, such as a quart fruit jar, and introducing a few live and dead eggs. Ideally, the dead or nonviable eggs should float, while the live or viable eggs slowly sink to the bottom. This technique is effective because dead trout eggs possess a lower specific gravity compared to live, eyed eggs (Anderson, 1964). If both types of eggs float, the solution is overly concentrated and should be diluted with water. Conversely, if both types of eggs sink, the solution is too dilute and requires the addition of more salt (Leitritz & Lewis, 1980).

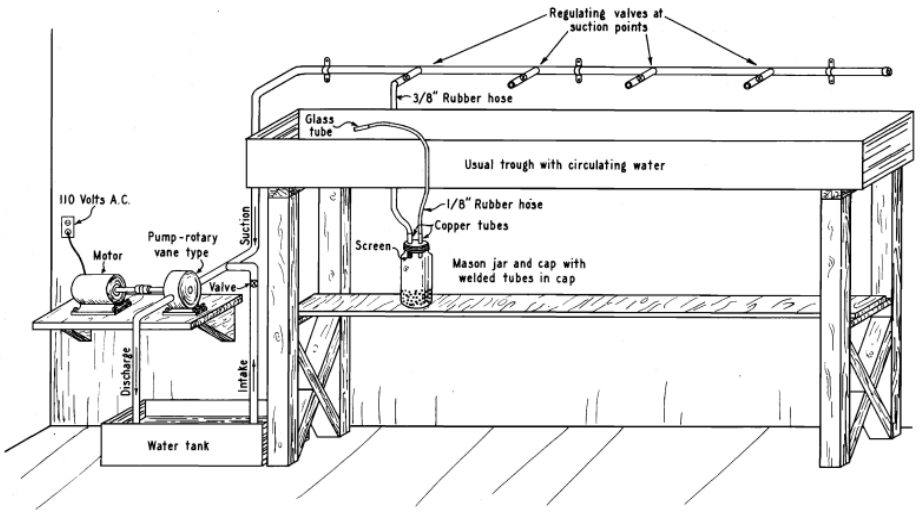

Figure 1.4 A power egg picker. An electric water pump creates a vacuum in a 2-quart mason jar through a pipeline and rubber hose system, enabling bad eggs to be drawn into the jar using a glass tube. The operator controls the speed via a valve, with the number of jars dependent on workspace. (McMullen, 1948)

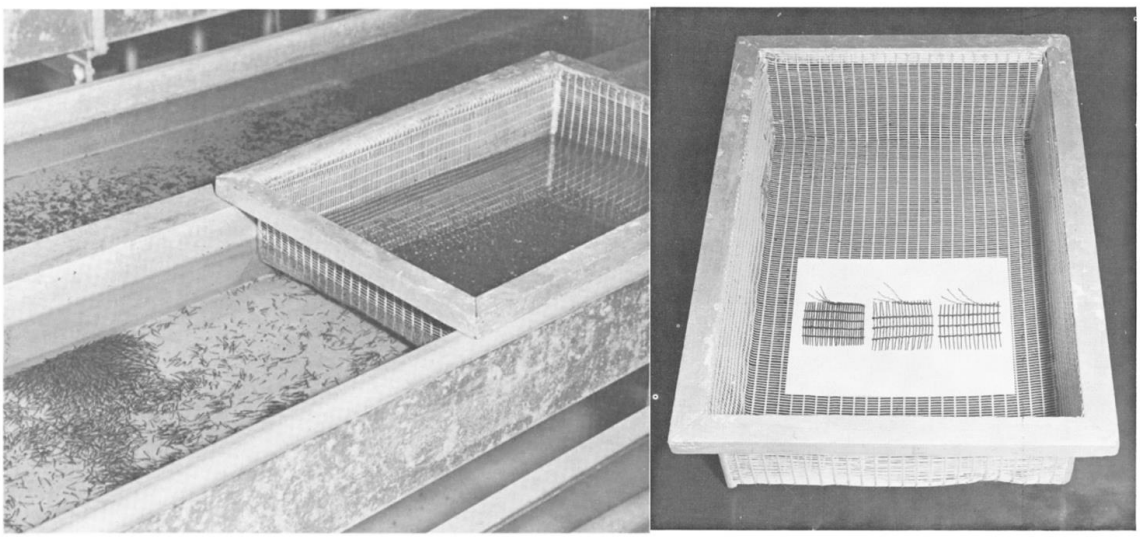

Figure 1.5 A trout egg basket. The conventional California egg basket measures 24 inches in length, 14.5 inches in width, and 6 inches in depth. Its outer frame, to which the basket is affixed, is crafted from wooden molding that is 1.5 inches by 0.75 inches in size. The mesh on the basket, specifically woven for this purpose, features rectangular openings designed to prevent round eggs from slipping through (Leitritz & Lewis, 1980).

Recently, more innovative techniques are being developed. In 2007, Eltink introduced the spray technique for an efficient separation of fish eggs from plankton. In order to accurately assess the abundance of ichthyoplankton in samples collected from the ocean, it is necessary to separate the fish eggs from the samples. Fish eggs are sprayed with a fine water jet, which removes plankton without damaging the eggs (Figure 1.6). This method has been successful for larger eggs and could potentially be modified to suit smaller eggs. In another study, Rohani et al. (2019) explored the application of artificial intelligence for the recognition of live and dead Rainbow Trout (*Oncorhynchus mykiss*) eggs. The study utilized image processing and machine learning algorithms to differentiate live from dead eggs. This method may significantly reduce the time and labor involved in egg separation, suggesting its potential application for other species including Delta Smelt and Longfin Smelt. However, the method does not provide a solution for separating dead eggs from the viable ones.

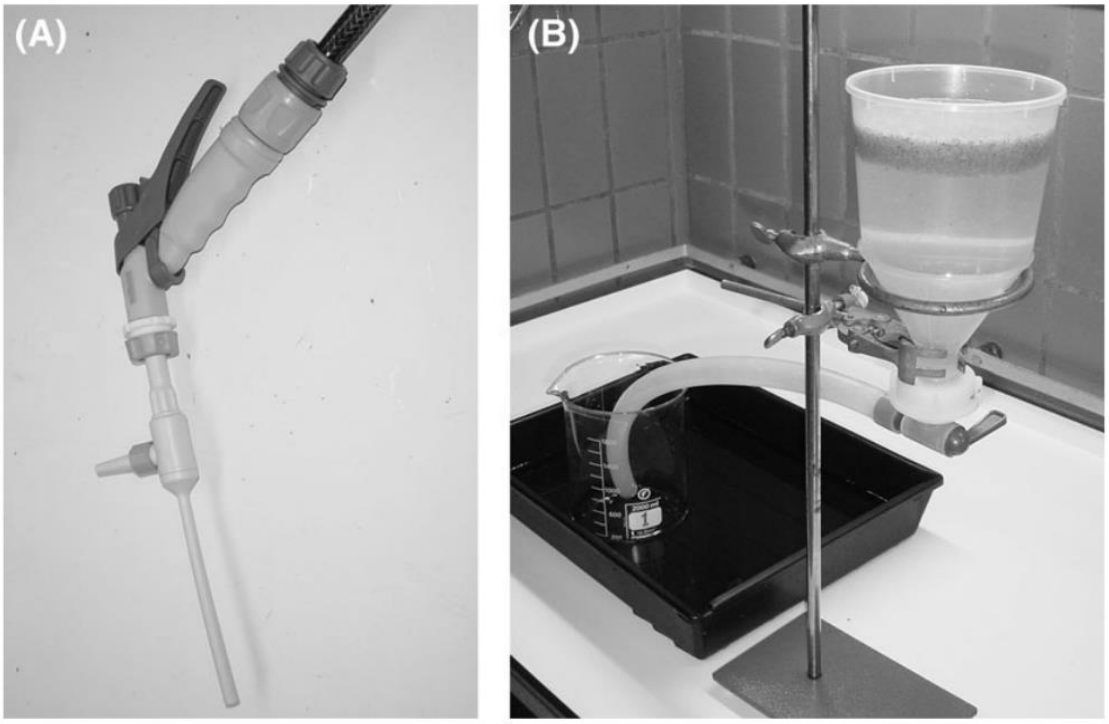

Figure 1.6 The spray technique. (A) The aerated seawater is produced by a water vacuum filter pump, which is connected to a spray gun. (B) The egg-sorting device for separating fish eggs from a plankton sample is a separating funnel. After spraying with aerated seawater, the plankton is floating, while the fish eggs sink to the bottom. The seawater with the fish eggs can be transferred into a 2-L glass beaker, which is placed in a black tray with water to enhance the visibility of the fish eggs. (Eltink, 2007)

Nevertheless, these methods were primarily devised for fish species that produce larger eggs. These applications for eggs from trout and salmon (egg size around 5.5 - 7 mm in diameter [Reid & Chaput, 2012].) are not directly applicable to Delta Smelt and Longfin Smelt due to the small size and adhesive properties of their eggs. Consequently, these methods are inefficient and difficult to adapt for these species. Therefore, there is a clear need for developing efficient egg separation methods that can address the unique reproductive characteristics of Delta Smelt and Longfin Smelt for the success of production programs.

1.5 Current Methods for Fish eggs Adhesiveness Removal

As highlighted in previous sections, the adhesiveness of Delta Smelt and Longfin Smelt eggs presents a substantial obstacle to the egg separation process. This trait constrains the application of conventional egg separation techniques typically employed for other species. Other species such as the sturgeons (*Pseudoscaphirhynchus spp.*, *Acipenser spp.* and *Huso spp.*), which also produce adhesive eggs, encounter similar challenges, limiting hatchery production (Siddique et al., 2016). At present, a variety of methods such as mechanical methods and chemical methods have been introduced in the aquaculture industry and scientific research to mitigate the adhesiveness of fish eggs.

For mechanical methods, methods include physically scouring them with clean water and treatment with abrasives such as clay, starch, charcoal, or bentonite (Demska-Zakęś et al., 2005). Common substances utilized for de-adhesion include mineral silt, milk, talcum, and blue clay or Fuller's earth. These substances exert a mechanical effect on the surface of the egg (Siddique et al., 2016). Monaco & Doroshov (1983) implemented de-adhesion processes by involving silt coating on White Sturgeon (*Acipenser transmontanus*) eggs. Meanwhile, Chebanov and Galich (2011) proposed that Fuller's earth is among the most effective substances for this purpose. However, the treatment durations, which range from 35 to 45 minutes for mineral silt and Fuller's earth, and 45 to 60 minutes for talcum powder, are still considered lengthy. The long time requirement could be perceived as a drawback. The application of these methods in hatchery settings can sometimes result in excessive labor requirements.

For chemical methods, studies suggest that egg adhesiveness can be managed by employing substances such as urea and NaCl solution, proteolytic enzymes, and tannic acid that chemically interact with the egg envelope (Siddique et al., 2016). The Woynarovich solution, a mixture of urea and NaCl, is the method most commonly employed in

aquaculture for dealing with egg adhesiveness (Woynarovich, 1962). Efforts have been made to enhance this existing technique by elevating the concentration of urea or incorporating additional ingredients such as fresh milk, powdered milk, or iodine solution (Siddique et al., 2016).

Proteolytic enzymes, according to the study (Siddique et al., 2016), have been proven to be the most effective method for eliminating adhesiveness in the eggs of several species, including Common Carp (*Cyprinus carpio*), European Catfish (*Silurus glanis* L.), and Tench (*Tinca tinca* L). The commonly used enzymes for elimination of egg stickiness are Alcalase, maxatase, trypsin, and α-chymotrypsin, which digest the proteinaceous layer and remove the adhesiveness.

For tannic acid and other substances, Kowtal et al. (1986) applied a solution consisting of 0.4% urea and 0.3% NaCl, followed by a brief bath in 0.1% tannic acid, to White Sturgeon eggs. The hatching rate observed was 57.7%, which does not significantly deviate from the results achieved through silt treatment (Monaco & Doroshov, 1983). Bouchard III & Aloisi (2002) compared the outcomes of treating Lake Sturgeon (*Acipenser fulvescens*) eggs with a urea-NaCl-tannic acid solution and Fuller's earth, noting hatching rates of 84.2% and 85.6%, respectively, at 14 days post-fertilization. The success of chemical egg treatments relies heavily on precise dosage and treatment duration. Incorrect dosages and extended treatment with tannic acid can elevate mortality rates (Chebanov et al., 2004). For instance, when eggs were immersed in a 0.15% tannic acid solution for durations of 60 and 90 seconds, it resulted in embryo mortality rates of 65% and 75%, respectively, for tench, and larval mortality rates of 85% and 95%, respectively (Kujawa et al., 2010). Conversely, in Pikeperch (*Sander lucioperca*), more favorable results were observed with a higher concentration of tannic acid (1 to 1.5 g/L) and a longer exposure time (5 minutes; Demska-Zakęś et al., [2005]).

To compare different adhesiveness removal methods, a study reviewed several different adhesiveness removal methods used for different species (Siddique et al., 2016). The study suggests that applying clay to some sturgeon eggs is a relatively straightforward and effective approach to reducing egg adhesiveness, though it is time-consuming. Also, unlike with cyprinids and other cultured fish species, the use of enzymes to remove egg adhesiveness in sturgeon has not yet been developed. In central Europe, powder milk is a common method of addressing this issue. Tannic acid can also be employed to eliminate egg adhesiveness. However, it has potential adverse effects, and inappropriate dosage or treatment duration can lead to high egg mortality. Proteolytic enzymes like trypsin, successfully used for Common Carp, European Catfish, and Pikeperch, could be beneficial for removing egg adhesiveness in sturgeon. The application of these enzymes could decrease treatment time and lower the risk of bacterial and fungal infestations.

1.6 Problem Statement

Numerous studies have explored the physical properties of fish eggs. They also examined various methods for fish egg separation and procedures to remove egg adhesiveness across different species. However, studies on the physical properties of Delta Smelt and Longfin Smelt eggs are limited. This gap in knowledge hinders the development of effective egg separation techniques for these two species. In order to develop an enhanced egg treatment process, one major challenge is the effective removal of egg adhesiveness. While an eggs adhesiveness removal process is included (Tsai et al., 2022a) before the egg separation process at the FCCL, it is not entirely effective, leading to difficulties in separating eggs and unnecessary losses. Alternative methods that address the adhesive property of the eggs also need to be explored for effective egg separation.

Additionally, due to the small size of the eggs of these two species, conventional egg separation methods such as power egg pickers developed for other species might not be directly applicable to these species. The lack of knowledge about the physical properties of the eggs further complicates the egg separation process, which currently relies on traditional methods that may not be optimized for these species. Thus, there is an urgent need for the development of new separation methods for effective and efficient egg separation, to improve the yield and quality of eggs produced for conservation and commercial purposes.

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CHAPTER 2

DEVELOPMENT OF AN EGG SEPARATING METHOD BASED ON THE DENSITY DIFFERENCE BETWEEN LIVE AND DEAD EGGS

2.1 Introduction

Among the fish egg separation techniques, including manual hand picking, pipette usage, mechanical egg sorter, and the salt flotation method (Leitritz & Lewis, 1980; McMullen, 1948; Smith, 1950), the last one utilizes the density differences between live and dead fish eggs, facilitating effective separation. Despite the small size of Delta Smelt (*Hypomesus transpacificus*) and Longfin Smelt (*Spirinchus thaleichthys*) eggs (approximately 1 mm in diameter, Romney et al., 2019), the efficacy of the salt flotation method may still be a potential tool. The simplicity and cost-effectiveness of the method further enhance its advantage among the other methods. However, given that most egg separation methods are primarily designed for large eggs (egg size around 5.5-7 mm in diameter [Reid & Chaput, 2012].), such as Salmonids (Leitritz & Lewis, 1980), their suitability for small egg species, such as Delta Smelt and Longfin Smelt, might be challenged. The unique characteristics of the eggs of these species, such as their small size and adhesiveness, might make existing methods inefficient or unsuitable. Furthermore, the density of the eggs of these species has not been previously investigated, and it remains uncertain whether the eggs can tolerate the salinity of the saline solutions and survive the process. This uncertainty creates a doubt on the applicability of the salt flotation method on them. These reasons necessitate an examination of the effectiveness of the salt flotation method when optimized and applied to these species.

The main focus of this chapter is to examine the potential applications of the salt flotation method on the fish egg separation and to evaluate the potential benefits and limitations when applied to Delta Smelt and Longfin Smelt eggs. Successful adaptation of

this technique to the unique characteristics of these eggs could lead to a more efficient separation process and aquaculture practices.

2.1.1 Density Separation Methods in different Industries

Density separation is a commonly employed method in different industries to separate objects with various densities (Gent et al., 2009). In the mining industry, for instance, density-based separators are frequently used to segregate ores from waste rocks. Dense medium separators using Dense Media Separation technology utilize a dense fluid that suspends particles of different densities, allowing low density particles to float and high density particles to sink (Tripathy et al., 2015). Another example, the Floatex density separator, also known as countercurrent bed separator, is a separator that uses differential particle settling rates to segregate particles according to size, shape, and density (Tripathy et al., 2015). These technologies are particularly effective for the separation of ores like diamond, coal, and iron. In the recycling industry, density separation is often used to sort different types of plastics (Gent et al., 2009; Serranti & Bonifazi, 2019). This type of plastics separation is based on whether particles sink or float in the separation medium. This effect is determined by the buoyancy exerted on the particles within a fluid. The efficiency of both sinking and floating methods is influenced by the size of the particles, as well as their aerodynamic or hydrodynamic shape (Richard et al., 2011). However, for materials with similar densities, using the flotation separation technique may be undesirable. For example, if air bubbles are attached to the surface of the particles, they may not be separated correctly (Kaiser et al., 2017). In other industries, these separation techniques also are part of a broader range of water treatment and recycling methods (Gupta et al., 2012). In the area of biofuel production, Milledge and Heaven (2013) highlighted the use of similar techniques for harvesting microalgae. They noted that for the micro-algal cells,

their similar density to the growth medium and their negative surface charge pose significant challenges to these processes.

In summary, density separation methods have been applied across various industries for different purposes. The technique spans applications from large, hard objects such as ores and rocks, to small objects like plastics and microalgae. This broad applicability indicates the potential to apply this technique to the egg separation of both Delta Smelt and Longfin Smelt. It also offers insights into how the method could be adapted to efficiently distinguish between live and dead eggs.

2.1.2 Epsom Salt Solution in Aquaculture: Applications on the Egg Separation

Separation of fish eggs in solutions with appropriate specific gravity (SG) has been investigated in the past. Solutions of table salt (NaCl) and granulated sugar ($C_{12}H_{22}O_{11}$) were recommended by the studies of Leitritz & Lewis in 1980 and Anderson in 1964 for this purpose. The principle behind separation by flotation is that hypertonic solutions cause unfertilized eggs to lose water, which in turn increases their specific gravity and causes them to sink to the bottom of the solutions (Weithman & Anderson, 1977). However, results have shown that table salt solution was not ideal for separating fish eggs, as it decreased the hatching rate after a certain duration of treatment (Fuss & Seidel, 1992; Weithman & Anderson, 1977). However, the mechanism of the decreased hatching rate remained unclear. From the study of Weithman & Anderson conducted in 1977, they noted that Rainbow Trout (*Oncorhynchus mykiss*) embryos exhibited hyperactivity and underwent a color change to a pale or whitish within 15 to 20 minutes of exposure to a table salt solution, leading to their eventual death. Additionally, granulated sugar solutions are not optimal because they are difficult to preserve; microbes grow rapidly in these solutions, potentially leading to egg contamination (Weithman & Anderson, 1977). In contrast, treatment with

Epsom salt ($MgSO_4$) solutions is not only cost efficient, but it has also been demonstrated to successfully separate Rainbow Trout eggs (egg size around 5.5-7 mm in diameter [Reid & Chaput, 2012].) without decreasing hatching rates (Weithman & Anderson, 1977). Given the cost-efficiency, safety, and effectiveness of Epsom salt solution treatments, this solution has been selected for use in the following experiments in this chapter.

In aquaculture, Epsom salt has been used for various purposes, including serving as a water treatment agent to improve water quality and to facilitate egg fertilization and separation (Chatakondi, 2019; Weithman & Anderson, 1977). Also, aquaculturists use Epsom salt to treat external monogenetic trematode infestations and external crustacean infestations in freshwater fish at all life stages (Smith, 1998). However, the egg separation methods developed thus far have primarily focused on larger eggs (greater than 5 mm in diameter), such as those of salmonids. Research on separating smaller or adhesive eggs, such as those of Delta Smelt and Longfin Smelt (egg size around 1 mm in diameter, Romney et al., 2019), has not been revealed. This chapter aims to explore the separation of smaller eggs by applying and expanding upon existing methods, with a focus on the eggs of Delta Smelt and Longfin Smelt.

2.2 Method and Materials

2.2.1 Fish Egg Production and Pre-treatment

Eggs from two fish species, Delta Smelt and Longfin Smelt, were utilized in this experiment. Eggs of both species were obtained from the UC Davis Fish Conservation and Culture Laboratory (FCCL, Byron, CA). Ripe fish of both species were identified using a standardized method based on tactile cues, which the maturity of a male or female fish was determined by gently applying pressure on their abdomen with fingers to check for the presence of milt and eggs, respectively. One male and one female were chosen to produce

each group of embryos. The spawning procedure followed the standard operating procedure at the FCCL (Ellison et al., 2023). For each group, ripe female and male were anesthetized using a 0.1% buffered tricaine methanesulfonate (MS-222, Syndel, USA) solution. After anesthetization, eggs were first striped from a ripe female directly into a dry plastic bowl. Once all the eggs were collected, milt from a ripe male was then stripped and directly applied to the eggs (Ellison et al., 2023). Water from the incubation system, 16.5°C and 0 ppt salinity for Delta Smelt (Lindberg et al., 2013) and 12°C and 0 ppt salinity for Longfin Smelt (Rahman et al., 2023) was introduced to the bowl, activating the fertilization process. After a period of one minute, the water was completely replaced. The bowl was then being placed in the water bath within the incubation system (Rahman et al., 2023).

Prior to the next step of the egg treatment process, the eggs must be incubated for a certain time. This incubation period is necessary for visually distinguishing between live and dead eggs (Tsai et al., 2021). The live eggs are characterized by their transparency, while the dead eggs appear opaque. (Mager, 1996; Romney et al., 2019; Tsai et al., 2021. Figure 2.1). The duration of this incubation varies between species: Delta Smelt eggs require three days incubation in the incubation system at a temperature of 16.5°C, while Longfin Smelt eggs require a longer period of six days at a cooler temperature of 12°C. Throughout the incubation process, the eggs received a daily treatment with 14.5 mL of Pond Rid-Ich Plus per liter of freshwater (Kordon, LLC, USA: active ingredients: 4.26% formaldehyde and 0.038% zinc-free chloride salt of malachite green) for one minute to minimize fungal growth (Baskerville-Bridges et al., 2005).

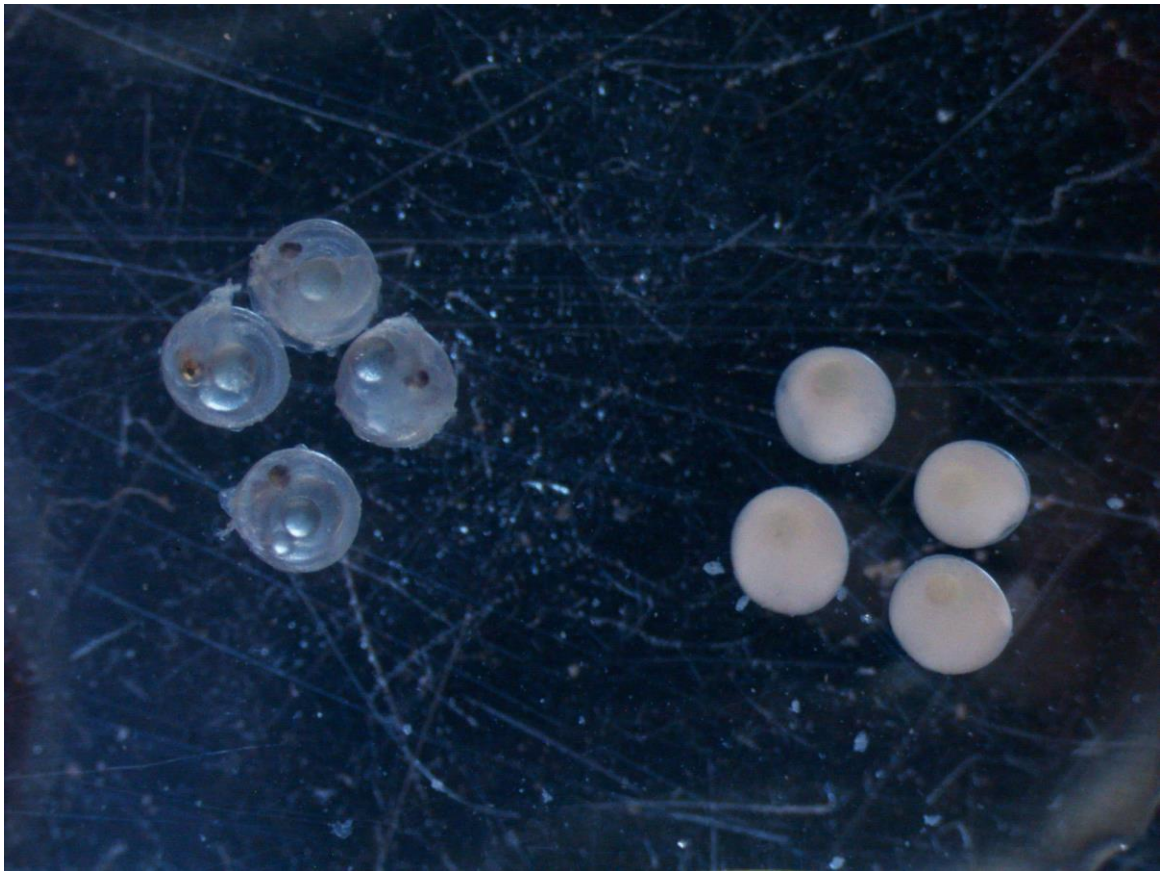

Figure 2.1 Delta Smelt eggs (photo taken on 7 days post fertilization). Opaque eggs on the right were classified as dead eggs, which encompasses unfertilized eggs. The relatively transparent eggs on the left were identified as live ones and successfully fertilized.

Following the first-step incubation, the eggs underwent several treatment processes including adhesiveness removal and disinfection before proceeding to the egg separation stage. These processes, as well as the duration of the treatments, were in accordance with the standard operating procedures for the manual egg separation process for both species at the FCCL. First, the adhesiveness of the eggs from both species was removed through a clay mixture (Bentonite clay, 16 g/L water; Sigma-Aldrich, USA) treatment (Lindberg et al., 2013). To remove the adhesiveness, the bentonite clay mixture (approximately 150 mL) was first prepared by blending 16 grams of bentonite clay powder with 1 liter of distilled water using a commercial countertop blender, ensuring a thorough mix. The bentonite clay mixture was then poured into a bowl with adhesive fish eggs, followed by a one-minute mechanical detachment process by gently rubbing the eggs off the surface with fingers.

After the adhesiveness was removed, the clay mixture was separated from the eggs using fish incubation system water and a fine mesh (500 micron) strainer. This was achieved by pouring the eggs and clay mixture into the strainer and rinsing it with incubation system water. This process effectively washed away the clay slurry, leaving the fish eggs in the strainer with the adhesiveness eliminated.

The eggs were then rinsed twice with incubation system water. Following the removal of adhesiveness, the eggs were subjected to an additional one minute treatment with 14.5 mL of Pond Rid-Ich Plus per liter of freshwater to further minimize fungal growth. These steps are the standard operating procedures at the FCCL before the manual egg separation process begins (Lindberg et al., 2013; Tsai et al., 2022). While these steps were consistent for both species, the temperature settings of the incubation system water used in these processes differed as described previously for the two species. After the completion of the processes, the separation tests were initiated. For the experiments in this chapter, only those eggs that have had their adhesiveness completely eliminated were selected for the experiments.

2.2.2 Experimental Design

2.2.2.1 Density Separation

In this study, the salt flotation method was tested by taking advantage of the density difference between dead and live fish eggs. This process involved separating the live eggs from the dead ones using Epsom salt solutions, within a certain range of SG. Eight Epsom salt solutions with the SG ranging from 1.04 to 1.18 (1.04, 1.06, 1.08, 1.10, 1.12, 1.14, 1.16 and 1.18) were prepared to find the settlement time needed for dead eggs and live eggs. This SG range was determined based on a preliminary experiment conducted on the Delta Smelt eggs. In those experiments, fish eggs were treated with Epsom salt solution with SG

ranging between approximately 1.00 to 1.22. Fish eggs were being observed to sink within the SG range from approximately 1.06 to 1.12. However, these experiments were designed to determine the SG range for the formal experiments, and the eggs were only being treated for 15 minutes. To ensure the accuracy of the experimental results, a SG range from 1.04 to 1.18 was established. Experiments were conducted separately for live and dead eggs. In each trial, which involves distinct SG values, a group of 30 eggs was placed in a beaker. This leads to a total of 64 groups for each species, calculated as follows: 8 SG values × 2 types of eggs × 4 replicates. The timer was started when the eggs were introduced to the solution and was stopped when all eggs had descended to the bottom of the beaker. A cutoff time of 1800 seconds (30 minutes) was established. Eggs that did not completely settle, i.e., they did not touch the bottom of the beaker within this cutoff time, were considered as not having completed the settling process.

2.2.2.2 Hatching Rate Determination

The assessment of hatching rates under Epsom salt solutions with three SG values and three treatment durations was conducted for eggs from both species. For each group, 20 live eggs were introduced to Epsom salt solutions with the SG values of 1.08, 1.10, and 1.12. Live eggs were recognized and chosen based on the method described previously. Three different treatment time periods, 10, 20, and 30 minutes, were applied to each different solution (3 SG values * 3 time periods * 3 replicates = 27 treatments (groups) for each species). Following the standard operating procedure at the FCCL, the pre-treatment processes including the bentonite treatment for eggs adhesiveness removal and the disinfection treatment were conducted before the treatment in the Epsom salt solutions. After the Epsom salt solution treatment, eggs were rinsed with incubation system water once, then transferred to a bowl containing 150 mL of incubation system water. The bowls

were floated on the water surface of the incubation system (a temperature-controlled recirculating system, Figure 2.2) to maintain the temperature throughout the incubation process. Following the FCCL incubation standard operating procedure, setting of the water temperature and incubation duration for the second-step incubation were 16.5°C and 7 days for Delta Smelt, and 12°C and 10 days for Longfin Smelt. Following these periods, hatching rates were examined. The hatching rates were determined by dividing the number of hatched eggs by the total number of incubated eggs, and the results were expressed as a percentage (%).

Figure 2.2 An egg incubation system at the FCCL. The system consists of upper incubation columns filled with fine sand, middle black buckets, and a water reservoir. Water is pumped from the reservoir to the columns, where regulated flow agitates the sand, ensuring fish egg movement and reducing fungal growth. As eggs hatch, larvae move to the buckets before water returns to the reservoir, creating continuous circulation. The water was maintained at 16.5°C for Delta Smelt and 12°C for Longfin Smelt.

2.2.3 Application on the hatchery-scale production

After confirming the hatching success in the small-scale experimental condition for both species, the Epsom salt solution with the SG value of 1.10 was chosen for the application on the hatchery scale fish egg production for both species at the FCCL. To confirm the efficacy and safety of the Epsom salt density separation method at a hatchery production scale, hatching rates were evaluated for both Delta Smelt and Longfin Smelt. The evaluations compared the hatching rates obtained using the Epsom salt separation against those using the traditional hand-picking separation. As described in Section 2.2.1 “Fish eggs Production and Pre-treatment", fish egg adhesiveness removal and pre-treatment processes followed the same procedures for the manual egg separation process for both species at the FCCL. The processes for mass production were identical across both species, with the exception of water temperature setting and incubation duration. These processes, along with the specific incubation conditions for each species, are detailed in Section 2.2.1 “Fish eggs Production and Pre-treatment". Furthermore, the mass production of the two species was carried out in distinct areas and systems within the FCCL. Specifically, Delta Smelt mass production took place in the Refuge Population Incubation Station (Figure 2.4), while Longfin Smelt mass production is conducted in the Egg and Live Prey Production Room (Figure 2.5).

In the hatchery-scale production application, all the eggs from a single cross, typically numbering over 1,000 eggs, were placed in a three-liter plastic pitcher for separation after the pre-treatment processes. This pitcher was filled with two liters of Epsom salt solution, which has a SG value of 1.10. The eggs were treated for a duration of 30 minutes. This specific SG value and treatment time have been demonstrated to be safe for the eggs in Section 2.3.2.1 “Small Scale Hatching Rate Experiment Analysis". After the 30 minutes treatment, the eggs that float on the surface of the solution, mostly live eggs, were poured

onto a fine mesh (500 micron) strainer to remove the solution. The eggs are then rinsed twice with incubation system water to eliminate any remaining Epsom salt solution. Remaining dead eggs are manually removed by a staff member using plastic pipettes (Fisherbrand™ Standard Disposable Transfer Pipettes, capacity: 7.7 mL; Molecular Bio Products Inc., USA). Finally, the live eggs are transferred to an incubation column (Figure 2.3) for incubation. For the data collection for both species, the egg spawning date, the total numbers of live eggs, and the hatching rate at 10 days post fertilization (DPF) for Delta Smelt eggs and 16 DPF for Longfin Smelt eggs were collected. Specifically, for Delta Smelt, the hatching rate data of Epsom salt separation from 18 groups of eggs, with average egg number of 4772 ± 1829 in every group against those using the traditional hand-picking separation for 18 groups of eggs, with average egg number of 3961 ± 2001 in every group were collected. For Longfin Smelt, the hatching rate data of Epsom salt separation from 12 groups of eggs, with average egg number of 1003 ± 709 in every group against those data from using the traditional hand-picking separation for 12 groups of eggs, with average egg number of 1060 ± 534 in every group were collected.

Figure 2.3 Incubation columns in the Egg and Live Prey Production Room at the FCCL. These columns are specifically designed for the incubation of Smelt eggs. During operation, fine sand fills the columns and water flows from the base of the columns, with the flow rate being controlled and modulated by the red valves as shown. This regulated water flow induces a gentle tumbling action for the eggs, which minimizes the likelihood of fungal contamination.

During the traditional hatchery-scale production of Delta Smelt, eggs from multiple Delta Smelt crosses were combined and placed into a single incubation column following the pre-treatment processes. Immediately after fertilization, the FCCL staff estimates the total number of fish eggs using a specialized technique. A glass pipette, connected to a rubber hose, is employed for this purpose. Eggs are introduced into the pipette along with the incubation system water. Once the water is carefully drained out through the rubber hose, each 1 mL marking on the pipette corresponds to an approximate count of 100 fish eggs (Baskerville-Bridges et al., 2005). For Delta Smelt, starting from 3 DPF, the number

of dead eggs was documented daily. By 10 DPF, the cumulative count of dead eggs and deformed larvae is subtracted from the initial total to determine the number of successfully hatched eggs. This allows for the calculation of the hatching rate at 10 DPF. For Longfin Smelt, all the procedures for the hatchery-scale production were identical except the total number of dead eggs was only documented once at 16 DPF, which is the time required for Longfin Smelt eggs to hatch.

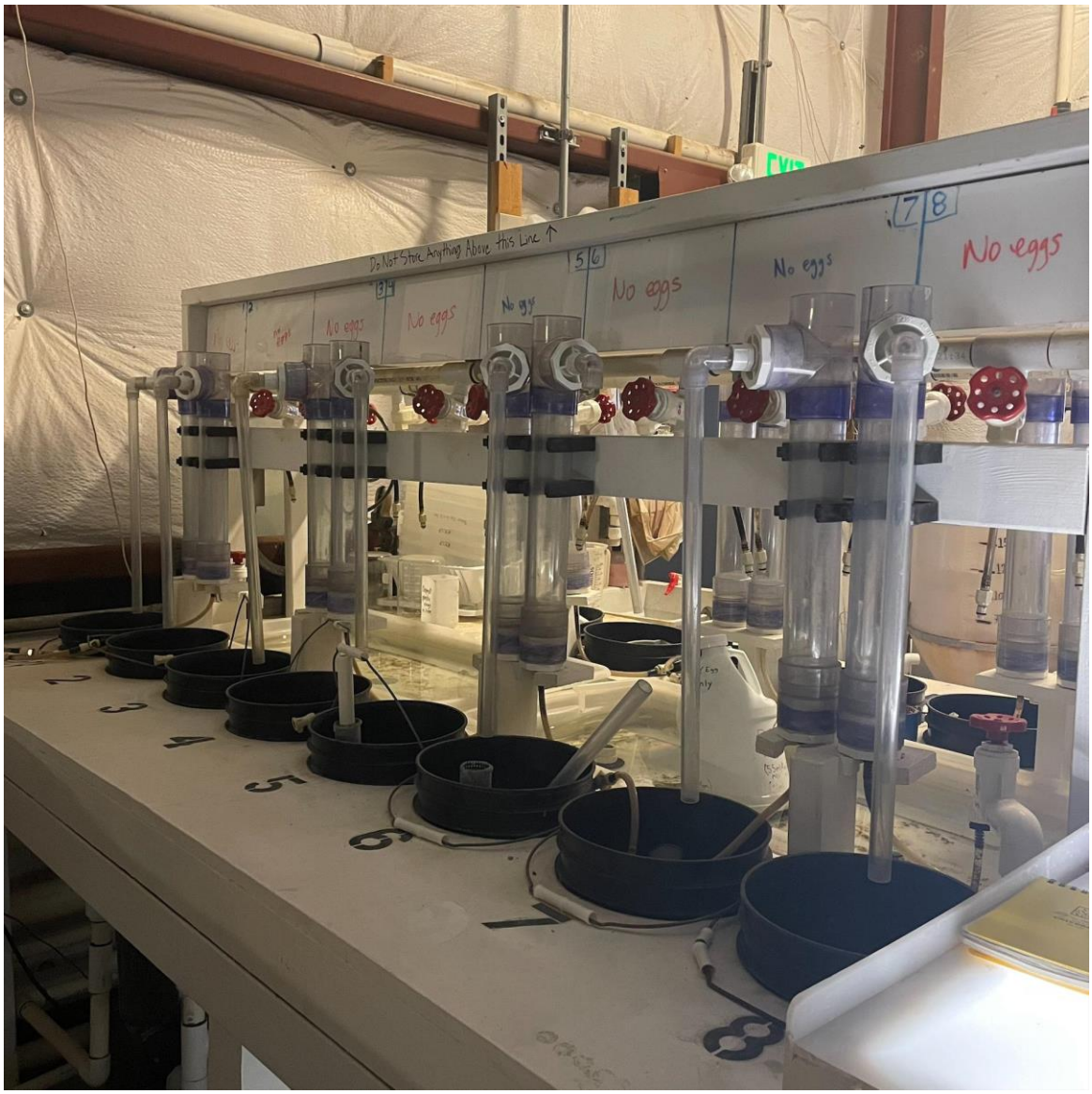

Figure 2.4 Delta Smelt incubation system for hatchery-scale production at the FCCL. The system consists of incubation columns filled with fine sand, black buckets, and a water reservoir.

Figure 2.5 Egg and Live Prey Production Room at the FCCL. The room houses three independent incubation systems as shown in Figure 2.2. They can operate independently, and their water temperatures can be adjusted to meet the specific needs of different species such as Delta Smelt and Longfin Smelt.

2.2.4 Scanning Electron Microscope Imaging

In order to understand the impacts to the egg surfaces from the bentonite clay treatment compared to the egg without treatment, the eggs of Delta Smelt were examined by utilizing a scanning electron microscope (SEM, Quattro S Environmental Scanning Electron Microscope, ThermoFisher, USA). Live and dead Delta Smelt eggs were examined at 3 DPF, and all the eggs were obtained from the same cross of Delta Smelt,

which the eggs were spawned only for SEM imaging. Images were captured under an accelerating voltage of 20 kv. Two samples were collected for SEM imaging. The first set of samples were obtained by directly scraping the live fish eggs from the bowl surfaces at 3 DPF. Although the procedures for fish egg production and pre-treatment remained consistent with those described in section "2.2.1 Fish Eggs Production and Pre-treatment", the use of bentonite clay for egg adhesiveness removal was excluded in this sample. For the second set of samples, the eggs underwent the complete fish egg production and pre-treatment processes, including the bentonite clay adhesiveness removal treatment. Live fish eggs were picked out afterward for the second sample.

Prior to the SEM imaging, the egg samples underwent a series of preparation steps. The fixation, dehydration, and gold coating process for the egg samples were conducted by the UC Davis Biological Electron Microscopy Facility. The eggs were initially fixed using a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. After fixation, the samples underwent dehydration using ethanol to remove any remaining moisture. This was followed by critical point drying, a process employed to preserve the structural integrity of the samples. Finally, before the SEM imaging, the dried samples were sputter-coated with gold.

2.2.5 Statistical Analysis

All statistical analyses were conducted using the R programming environment (R Core Team, 2023) version 4.3.1. A suite of libraries was employed to facilitate the various analyses, including: data processing and manipulation libraries: dplyr (Wickham et al., 2021), readr (Wickham & Hester, 2021), and tidyverse (Wickham, 2017), visualization libraries: ggplot2 (Wickham, 2016), scales (Wickham & Seidel, 2021), and ggh4x (Kleijn, 2021), statistical analysis libraries: car (Fox & Weisberg, 2019), pscl (Jackman, 2021),

rcompanion (Mangiafico, 2021), multcompView (Graves et al., 2019), emmeans (Lenth, 2021), multcomp (Hothorn et al., 2008), and rstatix (Kassambara, 2021), and other utilities: tcltk (Dalgaard, 2001) and extrafont (Chang, 2014).

2.2.5.1 Density Separation

The data were pre-processed to ensure that SG values were treated as factors, with levels ranging from 1.04 to 1.18. A Generalized Linear Model with a Poisson distribution was applied to analyze the relationship between the time taken for eggs to settle and the SG of the solution, taking into account the type of egg (live or dead). The model was specified as:

$$\text{Settlement Time} \sim \text{Egg Type} \times \text{SG}$$

The summary of this model revealed significant coefficients for all the predictor variables for both species. All the main effects and interaction terms were highly significant with p-values less than 0.001.

Following the model fitting, the ANOVA function was then used to perform a Type II analysis of variance on the model with a likelihood ratio test. Nagelkerke's pseudo R^2 was computed to gauge the goodness of fit of the model. The expected marginal means for the interaction between egg types and SG values were then calculated. Post hoc pairwise comparisons were performed on these marginal means, with adjustments made for multiple testing using the Sidak correction. Finally, Compact Letter Displays were generated to visually represent group differences based on the Sidak-adjusted post hoc tests.

2.2.5.2 Hatching Rate

2.2.5.2.1 Small-Scale Experiments Analysis

Prior to performing inferential statistics, two main assumptions of normality and homogeneity of variances were tested. The Shapiro-Wilk test was utilized to check the normality of the data, and the Levene's test was utilized to ensure that the variances across the different groups were approximately equal. In the assessment of hatching rates under Epsom salt solutions with three different SG values (1.08, 1.10, and 1.12) and three treatment durations (10, 20, and 30 minutes), we initially examined the normality of the data using the Shapiro-Wilk test. For the result of the hatching rate of Delta Smelt eggs, the test yielded a p-value of 6.095×10^{-6}, and for the hatching rate of Longfin Smelt eggs, the p-value was 5.78×10^{-6}. Both p-values fall well below the significance threshold of 0.05, thus the hypothesis of normality for both datasets were rejected. Next, the homogeneity of variances was tested through Levene's test. For Delta Smelt, homogeneity of variances across SG values and treatment times yielded p-values of 0.5354 and 0.7026, respectively. As for Longfin Smelt, homogeneity of variances across SG values and treatment times yielded p-values of 0.352 and 0.352, respectively. In both results, these p-values exceed the significance threshold of 0.05, suggesting that the variances across the groups are approximately equal. Given the violation of the normality assumption and the satisfaction of the homogeneity of variances assumption, the non-parametric Kruskal-Wallis test was conducted to assess the main effects of each factor of SG value and treatment time on the hatching rates.

2.2.5.2.2 Large-Scale Production Analysis

The data related to the hatching rates from the Epsom salt separation method and the conventional hand-picking method were loaded into R. The Shapiro-Wilk test was utilized

to check the normality of the data, and by confirming the non-normal distribution of hatching rate data, a Mann-Whitney U test was conducted to compare the hatching rates between the Epsom salt and traditional hand-picking method.

Initially, the normality of the hatching rate distributions for both species was examined using the Shapiro-Wilk test. For Delta Smelt, the p-value of 0.01005 was obtained from the Shapiro-Wilk test for the data distribution of the hatching rates from using the Epsom salt separation, while for the traditional hand-picking separation, the p-value obtained was 0.01178, suggesting that neither dataset followed a normal distribution. For Longfin Smelt eggs, the p-value of 0.8241 was obtained for the data distribution of the hatching rates from using the Epsom salt separation, suggesting that the dataset was not normally distributed. However, for the traditional hand-picking separation, the p-value obtained was 0.0004125, suggesting that the dataset was normally distributed. Given the results, one dataset (Epsom salt separation) appeared to be normally distributed and another (hand-picking separation) that was not. In such cases, it is prudent to use a non-parametric test. Thus, we proceeded with the Mann-Whitney U test to determine if there is a significant difference in hatching rates between the two methods.

2.3 Results

2.3.1 Density Separation

For both the datasets of Delta Smelt and Longfin Smelt, the Poisson regression analysis of the egg separation data suggests that both egg types and SG values are significant predictors of settlement time. Moreover, their interaction plays a crucial role, indicating that the effect of one predictor varies depending on the level of the other. The post-hoc analysis further illustrated these effects, highlighting specific contrasts that were significant.

For Delta Smelt, the settlement time differences between live and dead eggs showed significant differences at the SG values of 1.04, 1.06, 1.08, 1.10, 1.12, and 1.14 (Figure 2.6). For Longfin Smelt, the settlement time differences between live and dead eggs showed significant differences at the SG values of 1.04, 1.06, 1.08, 1.10, and 1.12 (Figure 2.7). For a more detailed understanding of the efficacy of each SG value in egg separation, differences in median settlement times between live and dead eggs were computed. In the case of Delta Smelt eggs, differences in median settlement times were observed for specific gravity values ranging from 1.04 (45.5 seconds), 1.06 (44 seconds), 1.08 (1,485 seconds), 1.10 (1,411.5 seconds), 1.12 (555 seconds), and 1.14 (283 seconds). For Longfin Smelt eggs, differences in median settlement times were observed for specific gravity values ranging from 1.04 (34 seconds), 1.06 (556 seconds), 1.08 (1,497 seconds), 1.10 (1,367 seconds), and 1.12 (997 seconds). No differences in settlement times were observed at specific gravity values of 1.16 and 1.18 for either species. Notably, at these two specific gravity values, all egg groups from both species consistently floated on the surface of the solution throughout the 30-minute experimental period. At the specific gravity values of 1.08, 1.10 and 1.12, all live egg groups from both species remained floating on the surface of the solution throughout the 30-minute experimental period, while dead eggs settled to the bottom within the 30-minute experimental period. Given this result, the SG value range from 1.08 to 1.12 was recommended for the large-scale hatchery fish eggs production for both Delta Smelt and Longfin Smelt.

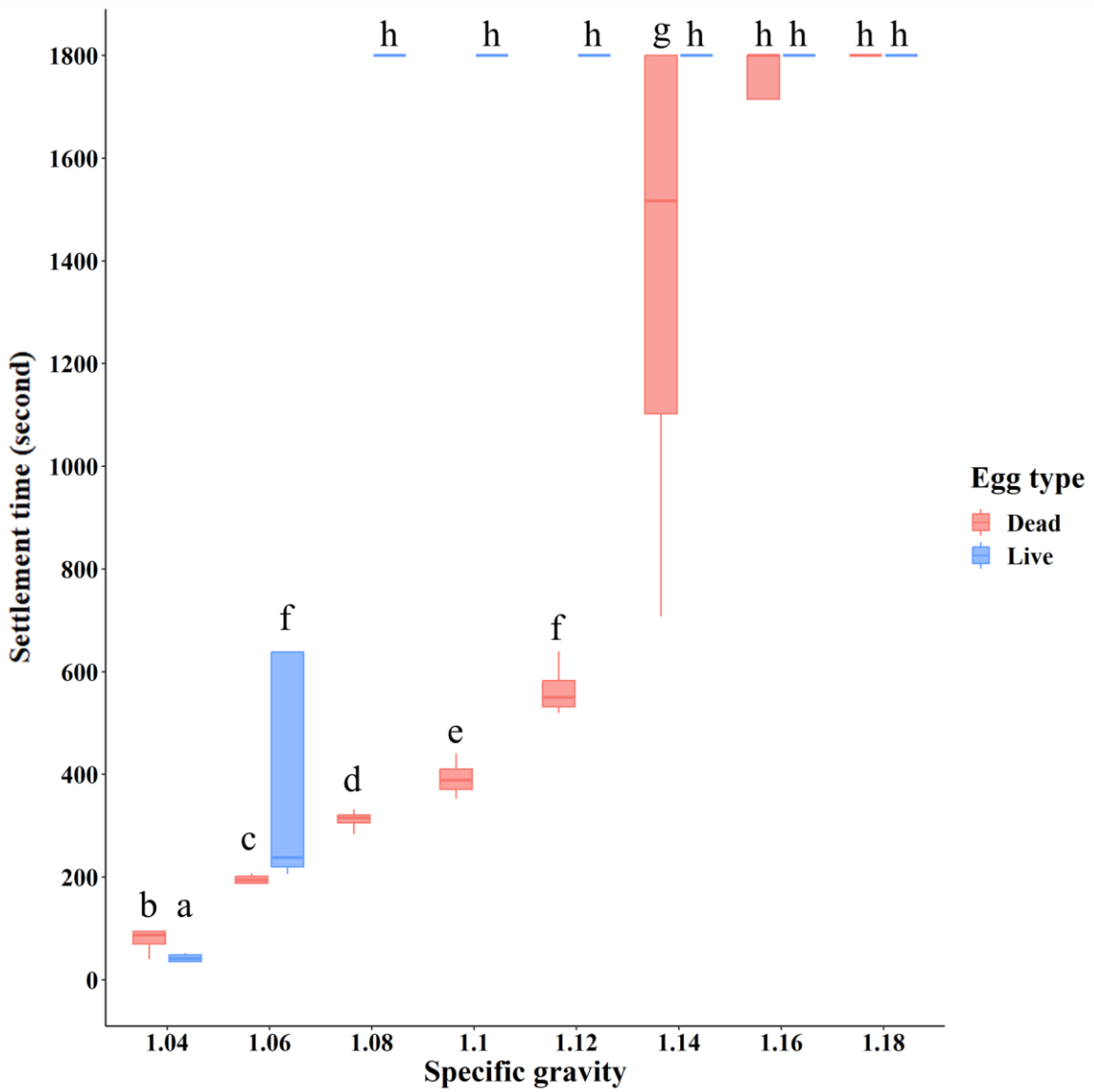

Figure 2.6. Settlement time for live and dead Delta Smelt eggs at various specific gravity values. Letters above the bars indicate significant difference ($p < 0.05$).

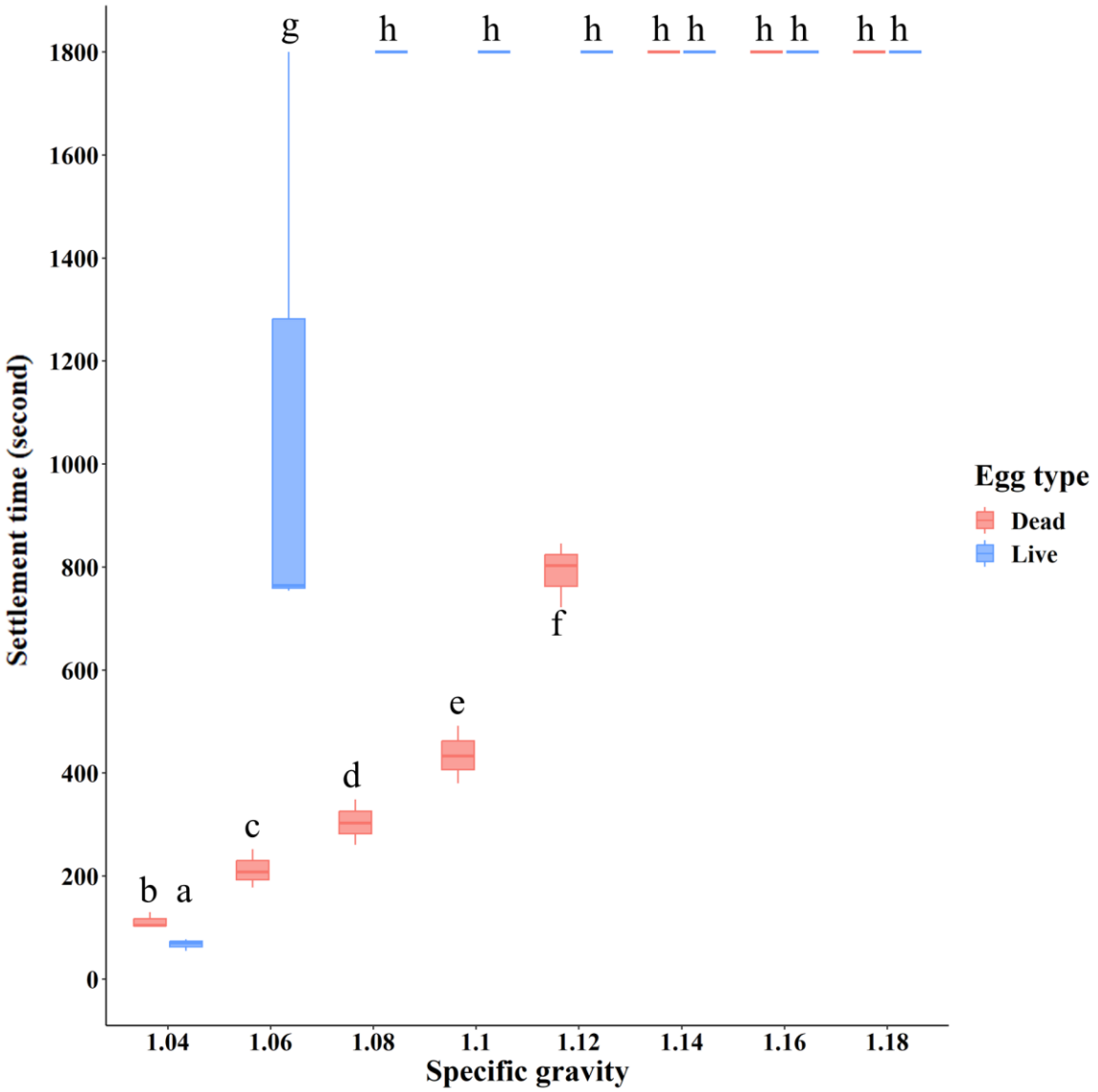

Figure 2.7. Settlement time for live and dead Longfin Smelt eggs at various specific gravity values. Letters above the bars indicate significant difference ($p < 0.05$).

2.3.2 Hatching Rate

2.3.2.1 Small Scale Hatching Rate Experiment Analysis

For Delta Smelt, there was no significant difference in hatching rates across the three SG values ($p = 0.7170$). When evaluating the effect of three different treatment times, the p-value was 0.6919. This result also suggested that there was no significant difference in hatching rates across the three treatment durations.

For Longfin Smelt, the result also suggested that there was no significant difference in hatching rates across the three SG values. For the effect of three different treatment times on hatching rate, the p-value was 0.3385, indicating no significant difference in hatching rates due to different treatment times.

In conclusion, for small scale experiments, there was no evidence suggesting that different SG values or treatment durations notably influenced the hatching rates of either the Delta Smelt or Longfin Smelt under the tested conditions (Table 2.1).

Table 2.1 Mean hatching rates of Delta Smelt and Longfin Smelt under different treatment conditions. No significant difference was found for both Delta Smelt and Longfin Smelt in hatching rates across the different SG values and treatment time.

		Hatching rate (Mean% ± SD)	
SG Value	Treatment time (minutes)	Delta Smelt	Longfin Smelt
1.08	10	100.00 ± 0.00	100.00 ± 0.00
1.08	20	93.33 ± 11.55	100.00 ± 0.00
1.08	30	93.33 ± 11.55	100.00 ± 0.00
1.10	10	83.33 ± 28.87	100.00 ± 0.00
1.10	20	93.33 ± 5.77	96.67 ± 5.77
1.10	30	96.67 ± 5.77	100.00 ± 0.00
1.12	10	93.33 ± 5.77	96.67 ± 5.77
1.12	20	96.67 ± 5.77	96.67 ± 5.77
1.12	30	100.00 ± 0.00	100.00 ± 0.00

2.3.2.2 Large-Scale Hatchery Production Hatching Rate Assessment

After confirming the hatching success in the small-scale experimental condition for both species, the Epsom salt solution with the SG value of 1.10 was chosen for the application on the hatchery scale fish egg production for both species. To confirm the

efficacy and safety of the Epsom salt density separation method at a hatchery production scale, hatching rates were evaluated for both Delta Smelt and Longfin Smelt. The evaluations compared the hatching rates obtained using the Epsom salt separation against those using the traditional hand-picking separation.

The Mann-Whitney U test was utilized to compare the hatching rates derived from the two methods for both species. The test resulted in a p-value of 0.001472 for Delta Smelt eggs and a p-value of 0.07343 for Longfin Smelt. For Delta Smelt, there was a significant difference in the hatching rates between the Epsom salt and hand-picking separation for the eggs ($p = 0.001472$, Figure 2.8). For Longfin Smelt, there was no significant difference in the hatching rates between the Epsom salt and hand-picking separation for the eggs ($p > 0.05$, Figure 2.9).

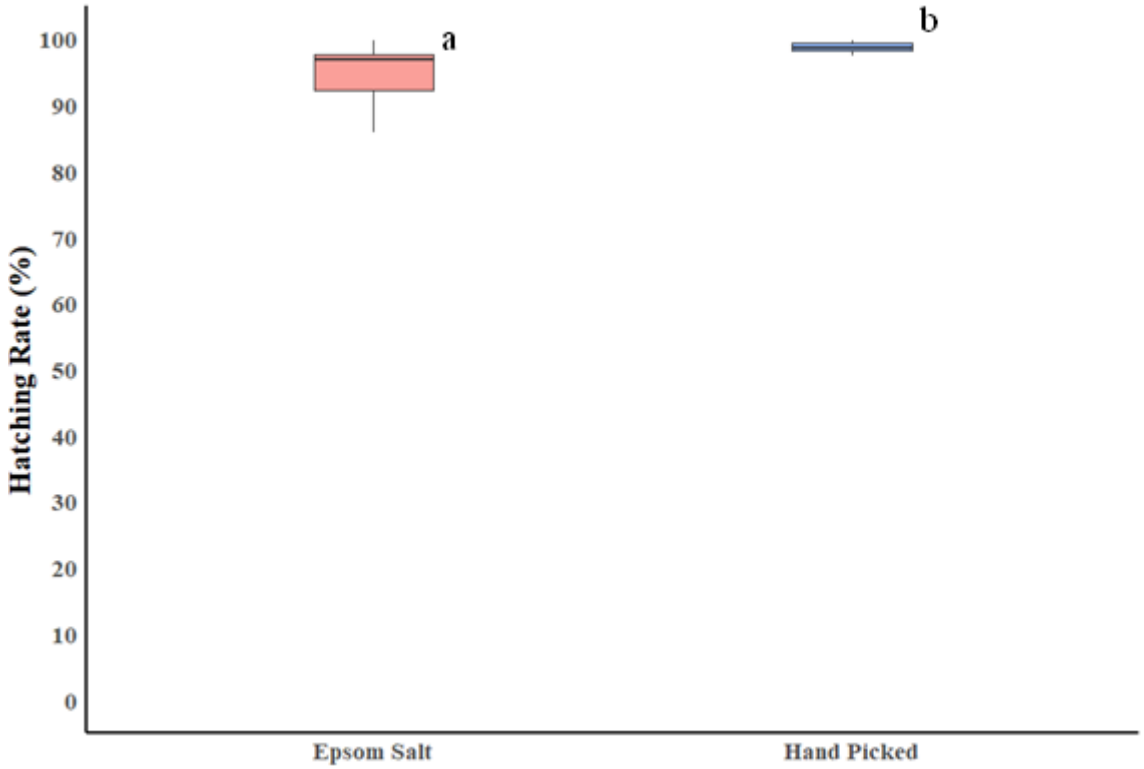

Figure 2.8. Delta Smelt hatching rates between two fish eggs separation methods in the setting of large-scale hatchery production. Letters above the bars indicate significant difference ($p < 0.05$).

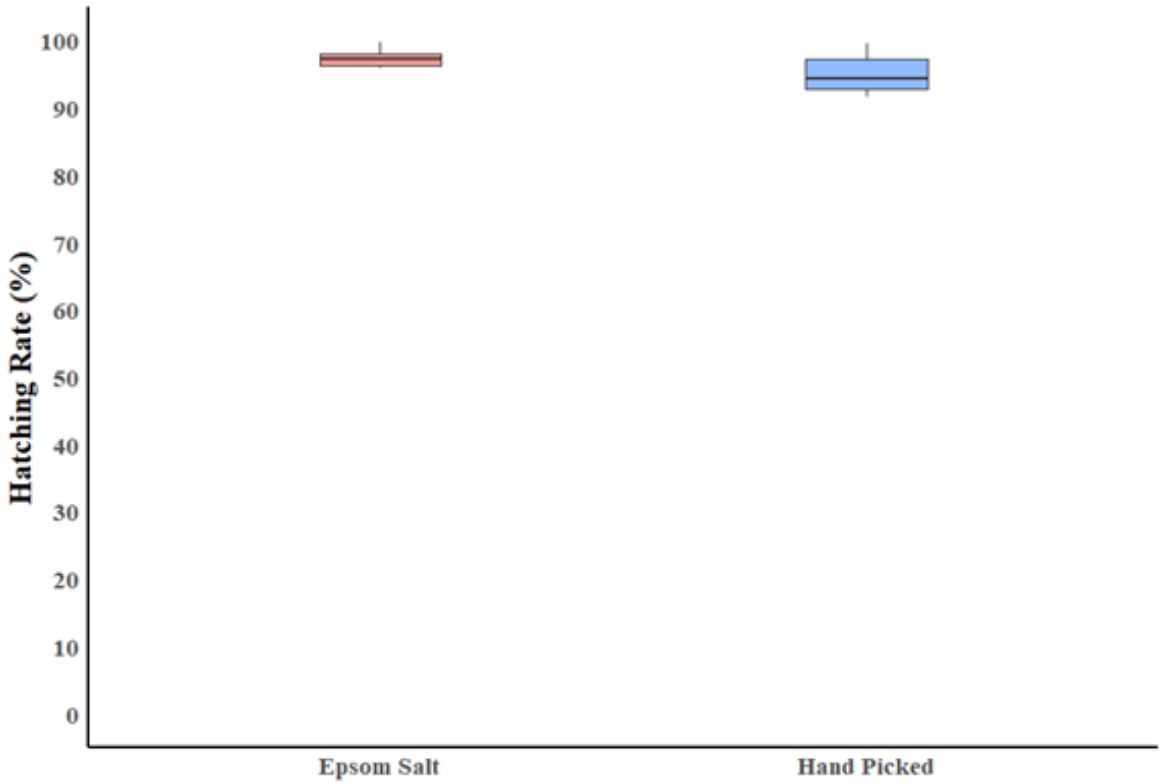

Figure 2.9. Longfin Smelt hatching rates between two fish eggs separation methods in the setting of large-scale hatchery production. No significant difference was found between two methods ($p > 0.05$).

2.3.3 Scanning Electron Microscope Imaging

Upon examination using the SEM, distinct surface structures were observed between Delta Smelt eggs treated with bentonite clay and those that were not (Figure 2.10). At magnifications of 2000x and 5000x, marked differences between the two egg samples were evident. The untreated egg showed a web-like structure accompanied by villi on its surface. In contrast, the egg treated with bentonite clay was completely covered by a debris layer, making the underlying egg surface barely visible. At an even higher magnification of 20000x, the details of the web-like structures and the surface debris became more visible.

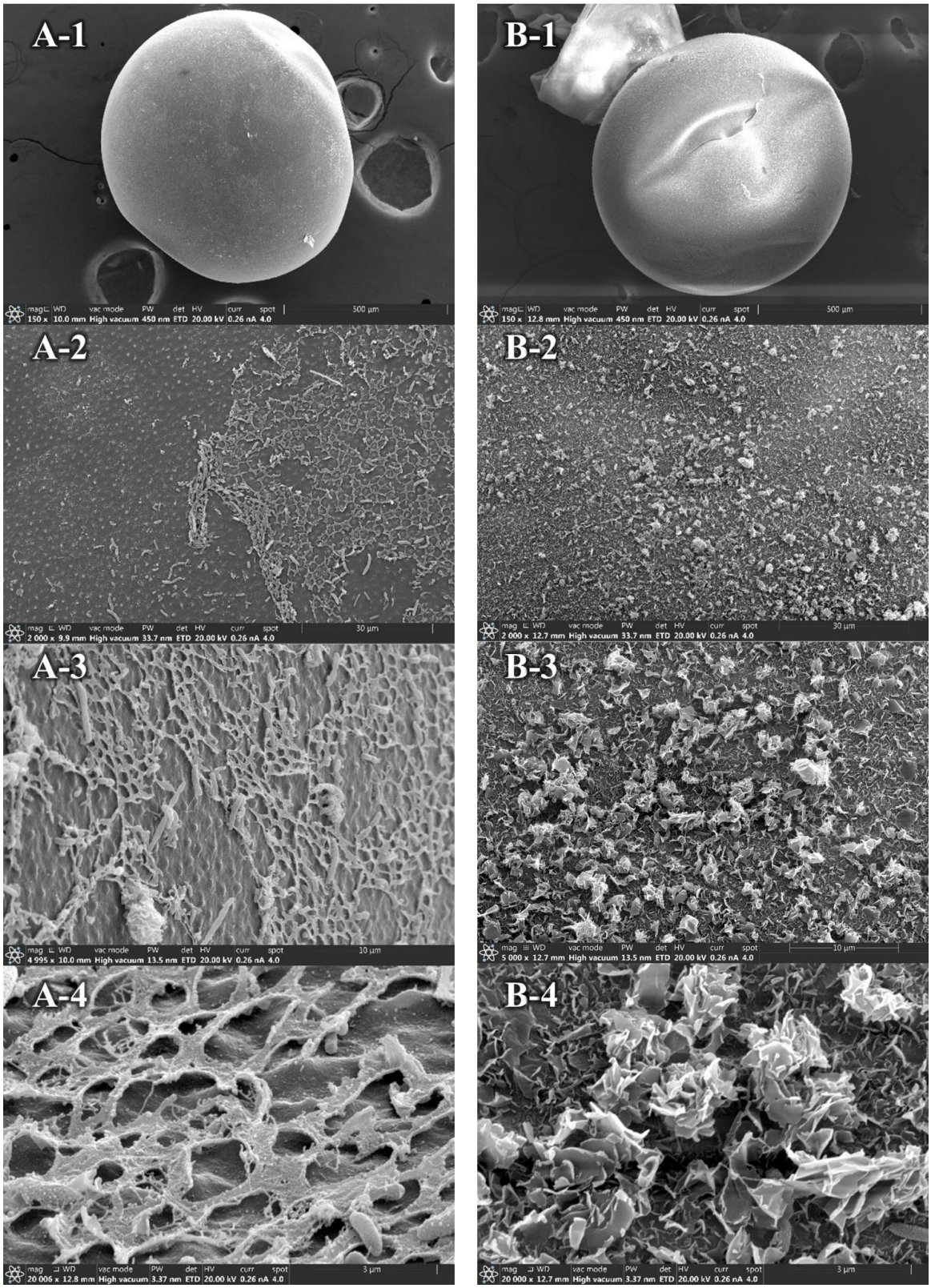

Figure 2.10 SEM images of Delta Smelt egg surfaces. Four images on the left (A-1 to A-4) show an egg without being treated with bentonite clay, while the four images on the right (B-1 to B-4) show an egg with bentonite clay treatment. The number '1' represents the magnification of 150x, '2' for 2000x, '3' for 5000x, and '4' for 20000x. Images were captured at an accelerating voltage of 20 kV.

2.4 Discussion

2.4.1 Fish Egg Separation in Small Scale Operations

At the FCCL, Delta Smelt eggs and Longfin Smelt eggs are handpicked in order to separate the dead eggs from the live eggs. This process requires a huge amount of time and labor and often causes eye fatigue. In this chapter, the alternative method of utilizing the density difference between the live and dead fish eggs was tested on both Delta Smelt and Longfin Smelt. From the results of the study, the density difference between live eggs and dead eggs was first observed. The specific gravity (SG) range that facilitated successful separation was identified to be between 1.08 to 1.12 for both Delta Smelt and Longfin Smelt. This finding is crucial, as it offers an efficient and potentially scalable approach for fish egg separation for both species. Results from these experiments provided several insightful revelations similarly to both species and suggested that this method could be implemented across varying scales of operations.

Rapid and complete separation between live and dead eggs is essential for efficiency and effectiveness, especially in hatchery settings. Given the close settlement times observed at SG values below 1.08, it would be practically challenging to ensure a consistent and reliable separation. On the higher end of the SG spectrum, the solution with an SG value of 1.14 presented its own set of challenges. For Delta Smelt eggs, some of the dead eggs did eventually settle to the bottom, but the time taken for this to happen exceeded 20 minutes. Such prolonged durations, especially when dealing with sensitive biological specimens like fish eggs, can introduce additional complications, such as increased exposure to solution and potential harm to live eggs. For Longfin Smelt, the SG value of 1.14 proved even more impractical as the dead eggs did not settle at all. This behavior suggests fundamental differences in the egg properties or structures between the two species, which might affect their buoyancy in the Epsom salt solution.

2.4.2 Application on The Hatchery Scale of Fish Egg Production

Scaling up any laboratory technique to a hatchery or large-scale production level usually comes with its own set of challenges, and the Epsom salt solution-based separation method for fish eggs is no exception. The method, while promising on the small-scale study, exhibited certain complexities when being applied at the hatchery scale. The transition from the controlled laboratory environment to the dynamic and large scale of hatchery production does require adjustments and optimizations.

First, one of the primary challenges was the difference in the separation time between small-scale and hatchery-scale operations. In large scale operations, the separation process took a slightly longer duration to achieve the desired result compared to the small-scale experiments. For all small-scale tests, when the SG value was set at 1.10, every dead egg took less than 600 seconds (10 minutes) to settle at the bottom of the beaker. Conversely, in large scale operations testing, some dead eggs failed to settle within the 10-minute treatment window. Given that the hatching rate remains unaffected by a 30-minute treatment in the Epsom salt solution with an SG value of 1.10, and to maintain consistency and assure the well-being of live eggs, the separation duration was standardized to 30 minutes. This duration, as validated by the hatching rate verification experiments, ensures the viability of the fish eggs and maintains a consistent separation rate.

Another challenge is the forming of egg clusters. The application of bentonite clay mixture as a solution for the removal of fish egg adhesiveness generally proved effective. The muddy consistency of the bentonite clay mixture, however, renders the eggs invisible during the treatment, making the process heavily reliant on the tactile skills and experience of the staff. The method involves manually rubbing the eggs off the surface of containers to ensure adhesiveness is removed, but due to the lack of visual confirmation, it is possible to inadvertently miss some eggs. Consequently, missed eggs can remain attached to one

another, resulting in the formation of egg clusters. These clusters pose challenges during the subsequent Epsom salt solution fish egg separation, as egg clusters often include both dead and live eggs, which the two types of eggs have been proved to behave differently in settlement behavior in the solution. In hatchery-scale fish egg production, it has been observed that these egg clusters cannot be effectively separated using the Epsom salt solution. Therefore, alternative adhesiveness removal methods that offer visibility during the process and can completely remove the adhesiveness are needed. Such methods might provide a better outcome of utilizing Epsom salt solution to separate fish eggs.

Using a bentonite clay mixture to remove fish egg adhesiveness not only results in debris formation but also further complicates the separation process due to the presence of this debris. This debris, present during the Epsom salt separation, could potentially interfere with the clean separation of live and dead eggs. The presence of such material introduces variables that could impact the consistency of the egg separation process. Utilizing a scanning electron microscope (SEM) to closely examine the egg surfaces, we identified the presence of bentonite clay debris (Figure 2.10). These findings suggest the challenges inherent to the bentonite clay treatment. While the bentonite clay was also employed in smaller, laboratory-scale settings and seemed not to substantially affect the outcomes, concerns about its potential impacts in larger-scale applications arise. The presence of this debris on the egg surfaces might influence the separation dynamics or even pose potential long-term effects on the eggs. These challenges underscore the need for alternative adhesiveness removal methods that provide visibility during the process and prevent debris deposition on the egg surfaces. Emphasizing the importance of this, Chapter 4 explored and tested various methods specifically for Delta Smelt eggs, aiming to refine and improve the efficiency of the egg separation process, especially at large scales.

In conclusion, the Epsom salt-based separation technique is promising but requires refinements for hatchery-scale applications. Streamlining pre-treatment processes, especially fish egg adhesiveness removal processes, could significantly enhance the efficacy of the new separation method at larger scales. As the scale of the production expands, refining these processes becomes more crucial.

2.4.3 Hatching Rate

While the effectiveness of using an Epsom salt solution for fish egg separation is evident from our study, it is important to understand the potential implications for the eggs. The safety of any egg separation approach can be measured by egg hatching rate, which offers direct information to the health and viability of the eggs, and success of the method. In this study, both the safety and efficiency were evaluated for the Epsom salt solution treatment across both small- and large-scale Delta Smelt and Longfin Smelt egg separation. The results were encouraging for the small-scale production trials. The hatching rates were not different across multiple SG values and treatment durations. Therefore, this new fish egg separation method was recommended for the trials on larger scale operations.

For a direct comparison, we focused on the new separation method using a single SG value of 1.10 and a fixed treatment duration of 30 minutes. When compared against the traditional hand-picking method, the Epsom salt fish egg separation yielded a slightly, but significantly, lower hatching rate of 95.44% compared to the 98.78% of the hand-picking method for Delta Smelt. However, for Longfin Smelt, the hatching rates yielded from the Epsom salt fish egg separation did not differ from the traditional hand-picking method. Given the prolific egg production capabilities of Delta Smelt and Longfin Smelt, wherein most of a single ripe female can easily produce over 2,000 eggs, this minor difference in

hatching rates might be offset by the significant efficiency gains of the new egg separation method.

At the FCCL, typical large-scale hatchery operations necessitate incubating around 700 live eggs for each cross of fish to ensure genetic diversity. Given the egg production capacity of Delta Smelt and Longfin Smelt, operational adjustments at the FCCL can readily accommodate this difference. By reserving a slightly larger number of live eggs for incubation, the same hatching yields can be achieved, thus leveraging the efficiency of the Epsom salt method without compromising on the overall hatching success. However, this reduced hatching rate in Delta Smelt eggs might require further investigation. The decrease in the hatching rate of Delta Smelt eggs could arise from potential effects of the Epsom salt solution on the eggs, variations in the estimation methods employed by the different FCCL staff, or other underlying factors. Studying more about the mechanisms causing this reduction will be a crucial topic of research in future studies.

2.4.4 Natural Breeding Environment of Delta Smelt and Longfin Smelt

In nature, Longfin Smelt is an anadromous fish species that inhabit throughout the San Francisco Estuary in California (Rahman et al., 2023). Longfin Smelt spawn in freshwater, migrate towards the ocean to forage and mature, and return to freshwater to spawn and begin the cycle again. On the other hand, Delta Smelt is a fish species that inhabit in the upper Sacramento-San Joaquin estuary in California, also spawn in freshwater and complete their life cycle within the freshwater-saltwater area (McAllister, 1963; Moyle, 1976; Moyle et al., 1992; Wang, 1986). Both species, while having different life cycles, share a common characteristic: the fragility of their populations in their natural habitats due to various anthropogenic impacts. The ecosystems they rely on have been heavily modified and are under constant pressures from water diversions, pollution, habitat destruction, and

invasive species (Moyle et al., 1989; Nichols et al., 1986; Williams et al., 1989). These pressures have led to significant declines in their populations, making it even more crucial to understand and optimize hatchery practices for their preservation. The natural salinity levels that these species experience during their life cycles should also be considered when developing egg separation and hatching techniques. While this study focused on the use of Epsom salt solutions for fish egg separation, it is crucial to recognize that such treatments should not drastically deviate from the natural osmotic conditions the eggs and larvae might experience in the wild.

In conclusion, while the results of our study in this chapter offer promising results for improving hatchery practices for Delta Smelt and Longfin Smelt, it is essential to always consider these practices in the context of the natural behaviors and environments of these species. Also, while the results have shown that this new egg separation method did not lower the hatching rate by too much, it is very important for future studies to understand the potential impacts to the fish after hatching.

2.5 Conclusion

The study presented in this chapter highlights the potential of the Epsom salt solution for fish egg separation. It offers an alternative to the traditional hand-picking method used at the hatchery. The manual technique, while effective, is time-consuming and often leads to worker fatigue. In contrast, the Epsom salt solution capitalizes on the density differences between live and dead eggs. Our findings point to an optimal specific gravity (SG) range of 1.08 to 1.12 for both Delta Smelt and Longfin Smelt. This discovery is vital for hatcheries where both efficiency and egg hatching rate are paramount. However, scaling the Epsom salt method to larger operations presents challenges. The separation time increases, and issues with the bentonite clay mixture, used for removing egg adhesiveness,

emerge. The clay leads to debris formation. Additionally, egg clusters form, complicating the separation process. These challenges emphasize the need for refining the technique, especially for large-scale applications. Hatching rates, essential for assessing any separation method's success, showed nuanced results. The new method had comparable rates for Longfin Smelt. However, Delta Smelt showed a slightly, but significantly, reduced rate. This difference requires further investigation. Lastly, it's crucial to consider the natural breeding environments of the fish. Both species face threats due to human impacts. Hatcheries play a significant role in their preservation. Any new method should enhance hatchery efficiency. But it should also align with the natural conditions and life cycles of the species. In essence, the Epsom salt-based fish egg separation shows promise. However, more research and refinements are needed, especially when thinking about the broader ecological context.

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CHAPTER 3

USING RAMSHORN SNAILS FOR THE REMOVAL OF NON-VIABLE FISH EGGS

3.1 Introduction

3.1.1 The Role of Ramshorn Snails in Delta Smelt Conservation

The use of animals in agriculture has been a topic of interest for researchers (Hasimuna et al., 2023). The applications of animals in the field can range from pest control to waste management. For aquaculture, one such animal that has been used for assisting aquaculture practices is the Ramshorn Snail (*Helisoma anceps*, Figure 3.1), a species of air-breathing freshwater snails, an aquatic pulmonate gastropod mollusk in the family *Planorbidae*. Ramshorn Snails are known for their ability to adapt to various environments and their omnivorous diet, which includes plant matter, detritus, and dead organisms (Hung et al., 2018). In aquaculture, this dietary habit can be utilized as the purpose of cleaning the environment by consuming dead organisms, excess feed, and algae. This behavior leads to a hypothesis that they may show a preference for consuming dead organisms, decomposed materials, and organic debris, rather than preying on living organisms. However, this is merely an assumption based on observations and their role in cleaning excess feed at the UC Davis Fish Conservation and Culture Laboratory (FCCL, Byron, CA, Hung et al., 2018).

The need for finding alternative ways for separating these eggs is crucial. At the FCCL, over 500,000 eggs of Delta Smelt (*Hypomesus transpacificus*) are spawned annually. After spawning, the egg adhesiveness was removed by using bentonite clay mixture (Lindberg et al., 2013), an important step after the adhesiveness removal is the separation of fertilized eggs from the unfertilized and dead ones. Dead or unfertilized eggs, prone to fungal infections like *Saprolegnia* (Leitritz & Lewis, 1980), can pose risk to the health of fertilized eggs, resulting in low hatching rates. Currently, the FCCL employs

manually separate the eggs by using plastic pipettes, which the process is labor-intensive and time-consuming. Efficient egg separation is crucial not only for successful hatching and larval production of Delta Smelt and Longfin Smelt (*Spirinchus thaleichthys*), but also for maintaining a genetically diverse population ready for reintroduction into the wild. This ensures the long-term health and adaptability of the species, contributing to the restoration of a balanced ecosystem in the Sacramento-San Joaquin estuary.

At the FCCL, Ramshorn Snail plays a crucial role in the cultivation of larval and juvenile Delta Smelt and Longfin Smelt (Hung et al., 2018; Tsai et al., 2022). They are regularly employed to remove excess feed and algae and carry out cleaning tasks in the fish tanks (Hung et al., 2018). This method facilitates the removal of excess algae, uneaten food, and feces from the tank, which is advantageous when manual removal is not ideal due to the potential risk of causing harm to the fish or accidentally removing some of the larvae (Hung et al., 2018). Since snails continuously consume the organic matter in the tanks, they also decrease the chance of the growth of fungus and promote survival of the juvenile fish (Hung et al., 2018). However, the specific feeding preferences and behaviors of Ramshorn Snail, especially their potential consumption of live organisms such as Delta Smelt eggs, are not well documented. Moreover, their potential role in the fish egg separation process, particularly in distinguishing between live and dead eggs, is unknown and is the primary focus of this study. In this chapter, a novel approach of utilizing Ramshorn Snails for fish egg separation was tested. And because of these existing and potential feeding behaviors of Ramshorn Snails, the idea of leveraging these snails to selectively remove dead fish eggs was proposed to be tested, potentially adding an ecological, sustainable, and more efficient dimension to the fish egg separation process.

Figure 3.1 A Ramshorn Snail (*Helisoma anceps*) at the FCCL. Within the facility, the Ramshorn Snails serve a critical role as a fish tank cleaner, proficiently eliminating excess feed and algae accumulations. These snails achieve their cleaning function without posing any harm to the sensitive fish larvae present in the fish tanks.

3.1.2 Selective Behavior Towards Dead Eggs in Animals

In the animal kingdom, there are some examples of selective behavior towards dead eggs. One such example is the Mimic Poison Frog (*Ranitomeya imitator*). In this species, females, cued by the calls of their male partners, feed their tadpoles with unfertilized eggs. This behavior demonstrates a form of selective behavior towards dead eggs in the context of parental care (Moss et al., 2022). In the realm of terrestrial animals, specific bird species have been observed to selectively remove or discard dead eggs from their nests based on certain factors such as size, color, or the overall condition of the eggs (Davies & Brooke, 1989). This behavior, referred to as egg discrimination, is believed to be an adaptive response aimed at minimizing the risk of investing parental care in non-viable eggs or eggs laid by brood parasites (Davies & Brooke, 1989). In the context of gastropods, there is a

lack of specific studies that directly address the selective behavior towards dead eggs. However, the general feeding behavior of many gastropods, including their preference for certain types of food including organic matters from dead organisms, may suggest the potential for a dietary preference towards dead fish eggs (Benjamin, 2012; Yamane & Gilman, 2009).

3.1.3 Olfactory Navigation and Food Detection of Gastropods

One of the primary mechanisms gastropods use to detect food is through olfactory cues, guiding their navigation behavior. They utilize these cues for various purposes, such as locating prey, avoiding predators based on kairomones, or identifying potential mates using pheromones (Wyeth, 2019). Wyeth (2019) provides a comprehensive review of the navigational behaviors observed across major aquatic gastropods. The study suggests that gastropods likely employ chemotaxis or odor-gated rheotaxis, or a combination of both, during olfactory-based navigation. Despite the significant insights into how turning behavior is triggered by contact chemoreception, there remains a gap in understanding how navigation relative to distant odor sources is controlled in gastropods (Wyeth, 2019). The diet of many gastropods includes decaying organic matter (Hung et al., 2018), which could potentially include dead or decaying eggs.

Ramshorn Snails, the target species in this study, is a good example of these feeding habits. Although the primary food source of these snails is algae, studies also suggested that they also graze on detritus (Burch, 1989, 1992; Weber & Lodge, 1990). These studies also propose that chemoreception might be the mechanism these snails use to detect food with different properties. Bovbjerg (1968) investigated the feeding and dispersal behavior of four lymnaeid snail species, which are also believed to use chemoreception for food detection. The study found that while the snails did not respond to diffusible plant-based

food substances, they did react to diffusible animal-based food substances, moving towards the source. Interestingly, despite the common assumption that these snails are strictly herbivorous, the study revealed that they are actually omnivorous. Their movement towards diffusible animal substances suggests a distant chemoreception to animal matter, thus strengthening the hypothesis for this study that Ramshorn Snails may exhibit a preference for dead fish eggs over live ones.

3.2 Methods and Materials

3.2.1 Fish Eggs Production and Pre-treatment

In this chapter, the eggs of Delta Smelt were utilized for the experiments. The methods for the fish egg production and pre-treatment processes followed the standard operating procedures established by the FCCL (Lindberg et al., 2013). Mature fish were identified by gently applying pressure to their abdomen to confirm the presence of eggs and milt (Ellison et al., 2023). Each batch of eggs was obtained from a single female and fertilized with the milt from one male. To carry out the fertilization, eggs were initially extracted from a mature female into a dry bowl by applying pressure on the abdomen. Immediately after the eggs were stripped from the female, milt from a mature male fish was extracted using the same method and directly added to the eggs. The eggs were then gently mixed with a plastic stick to ensure a thorough combination of the eggs and milt. Subsequently, around 150 mL of water from the Delta Smelt egg hatching system was added to the bowl to initiate the fertilization process. Upon contact with water, the eggs became adhesive. The water in the bowl was fully replaced once after one minute, and the bowl was then floated in a constant 16.5°C water bath within the sump of the incubation system for a period of 3 days for the first-step incubation.

Following the fertilization process, the fish eggs underwent several treatment processes including adhesiveness removal and disinfection before proceeding to the egg separation stage. These processes, as well as the duration of the treatments, were in accordance with the standard operating procedures for the manual egg separation process at the FCCL (Lindberg et al., 2013). First, the adhesiveness of the eggs was removed through a clay mixture (Bentonite clay, 16 g/L water; Sigma-Aldrich, USA) treatment. To remove the adhesiveness from the eggs, the bentonite clay mixture (approximately 150 mL) was first prepared by blending 16 grams of bentonite clay powder with 1 liter of distilled water using a commercial countertop blender. The bentonite clay mixture was then poured into a bowl with adhesive fish eggs, followed by a one-minute mechanical detachment process by gently rubbing the eggs off the surface with fingers. After the adhesiveness was removed, the clay mixture was separated from the eggs using Delta Smelt incubation system water (16.5°C and 0 ppt salinity, Lindberg et al., 2013) and a fine mesh (500 micron) strainer. This was achieved by pouring the eggs and clay mixture into the strainer and rinsing it with incubation system water. This process effectively washed away the clay slurry, leaving the fish eggs in the strainer with the adhesiveness eliminated. The eggs used in these experiments were at the stage of 3 days post-fertilization (DPF) and had undergone these pre-treatment processes to fully remove their adhesiveness, and live and dead eggs were distinguished visually (Tsai et al., 2021). In this chapter, we utilized three Delta Smelt crosses for the eggs production. For each cross of Delta Smelt, three experimental groups and one control group were established.

3.2.2 Ramshorns Snails Culture and Selection

Adhering to the FCCL operation procedure, large size snails (shell diameter exceeding 1.3 cm, [Hung et al., 2018]) were specifically selected for the experiment. These

large size snails are also the snails being utilized for cleaning the fish tanks at the FCCL. From the larger snail rearing tanks (5 foot in diameter, Figure 3.2) which are specifically designated for the cultivation of these snails, 40 large snails (16.70 ± 0.60 mm in shell diameter) were randomly selected. These selected snails were then transferred to a 5-gallon HDPE bucket (The Home Depot, Inc., USA), which was filled with approximately 4 gallons of Delta Smelt incubation system water. Snails were kept indoors in this bucket at room temperature (23°C) for a period of 48 hours prior to the experiments. Throughout this preparatory period, the snails were not provided with any food. After the first 24 hours, the water in the bucket was completely replaced, and any feces present were removed. After the 48-hour period in the bucket, 18 snails were arbitrarily selected from the initial group of 40 to form a total of 9 experimental groups (i.e., 2 snails per experimental group).

Figure 3.2 A Ramshorn Snail rearing tank at the FCCL. With a diameter of 5 ft, the tank is equipped with an airstone for aeration. Fresh water is continuously introduced into the tank via a pipe positioned on its edge, while water exits the tank through a central pipe. To ensure that the snails do not get carried out with the outgoing water, this central pipe is encased in a mesh.

3.2.3 Experimental Design

In this study, eggs from the three distinct Delta Smelt crosses were utilized separately. For each cross, 3 experimental groups and 1 control group were established, which made the total group number of 12 (i.e., 3 crosses × [3 experimental groups + 1 control group]). Each experimental group consisted of one bowl containing 100 mL of Delta Smelt incubation system water and 30 Delta Smelt fish eggs at 3 DPF, constituted with 15 live eggs and 15 dead eggs. Two large-sized Ramshorn Snails were introduced into each bowl.

The control group, on the other hand, consisted of only 15 live eggs and 15 dead eggs in 100 mL of incubation system water in the bowl, with no snails present. For the environmental setting of the experiment, all bowls were placed in a water bath within the sump of the incubation system (Figure 3.3) throughout the experiment, ensuring that the water in the bowls did not come into direct contact with the water in the incubation system. A consistent temperature of 16.5°C was maintained throughout the experiment by a water chiller attached to the incubation system. The bowls were taken out from the water bath for 5 minutes once every 24 hours in order to perform water change to ensure optimal conditions and to calculate the amount of the eggs in the bowl. This was done by carefully pouring out all of the old water from the bowls and adding fresh incubation system water. Live and dead eggs were identified visually following the standard method established at the FCCL (Tsai et al., 2021). During the experiment, live eggs and dead eggs were counted and documented once a day (24 hours) starting from 3 DPF and ended at 7 DPF.

Figure 3.3 The sump of the Delta Smelt incubation system. To maintain a constant temperature, water circulates within the system and the water temperature is regulated by a water chiller. The water within these bowls is refreshed every 24 hours using water from the sump.

3.2.4 Scanning Electron Microscope Imaging

In order to understand the differences between the surfaces of the dead fish eggs and live fish eggs, the eggs were examined by utilizing a scanning electron microscope (SEM, Quattro S Environmental Scanning Electron Microscope, ThermoFisher, USA). Live and dead eggs were examined at 3 DPF, and all the eggs were obtained from the same cross of Delta Smelt, which the eggs were spawned only for SEM imaging. Images were captured

under an accelerating voltage of 20 kv. Samples for the SEM imaging were collected by gently scraping the fish eggs from the bowl surfaces at 3 DPF. While the procedures for fish egg production and pre-treatment were consistent with those outlined in section “3.2.1 Fish Eggs Production and Pre-treatment", the use of bentonite clay to remove egg adhesiveness was omitted. For the purpose of SEM imaging, a plastic stick was employed to directly remove the live and dead eggs from attaching to the bowl surfaces.

Prior to the SEM imaging, the egg samples underwent a series of preparation steps. The fixation, dehydration, and gold coating process for the egg samples were conducted by the UC Davis Biological Electron Microscopy Facility. The eggs were initially fixed using a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. After fixation, the samples underwent dehydration using ethanol to remove any remaining moisture. This was followed by critical point drying, a process employed to preserve the structural integrity of the samples. Finally, before the SEM imaging, the dried samples were sputter-coated with gold and were ready for the SEM imaging.

3.2.5 Statistical analysis

All statistical analyses were conducted using the R programming environment (R Core Team, 2023) version 4.3.1. A suite of libraries was employed to facilitate the various analyses, including data processing and manipulation libraries: dplyr (Wickham et al., 2021), readr (Wickham & Hester, 2021), visualization libraries: ggplot2 (Wickham, 2016), and other utilities: tcltk (Dalgaard, 2001) and extrafont (Chang, 2014).

Before applying any statistical tests on the data, the normality of the data was first tested. The Shapiro-Wilk test was conducted for testing data normality and applied separately for the rate of change in live and dead egg counts from 3 DPF to 4 DPF. For assessing the rate of change in egg counts, the distribution of live and dead eggs was first

tested for normality at each DPF. The Shapiro-Wilk test indicated that on the 4 DPF, the rate of change for live eggs was significantly deviated from a normal distribution (p-value = 0.0002313). Due to the non-normal distribution of the data on certain days, the non-parametric Mann-Whitney U test was chosen to compare the rate of change in numbers between live and dead eggs for the first 24 hours of the experiment (3 DPF to 4 DPF).

3.3 Results

3.3.1 Consumption of Fish Eggs by Ramshorn Snails

The main objective of this study was to determine whether Ramshorn Snails exhibit a preference for consuming dead Delta Smelt eggs over live ones. Results from the Mann-Whitney U test revealed a distinct pattern. From 3 DPF to 4 DPF, a significant difference was observed in the egg counts changing rates between live and dead eggs (p-value = 0.009091). Dead eggs were consumed at a significantly faster rate compared to live ones (Figure 3.4).

Of particular note, from 5 DPF to 7 DPF, most experimental groups entirely lacked dead eggs (Figure 3.5), with the exception of two group where one dead egg was observed on 6 DPF (72 hours after the experiment started), and one group where one dead egg was observed on 7 DPF (96 hours after the experiment started), likely resulting from the death of live eggs. In control groups, the amount of live fish eggs decreased from 3 DPF to 7 DPF, with the amount of dead fish eggs increased from 3 DPF to 7 DPF (Figure 3.5). Notably, during the process of utilizing Ramshorn Snails for Delta Smelt egg separation, some eggs were identified within the feces of the snails (Figure 3.6). This further proved that these snails consumed the fish eggs.

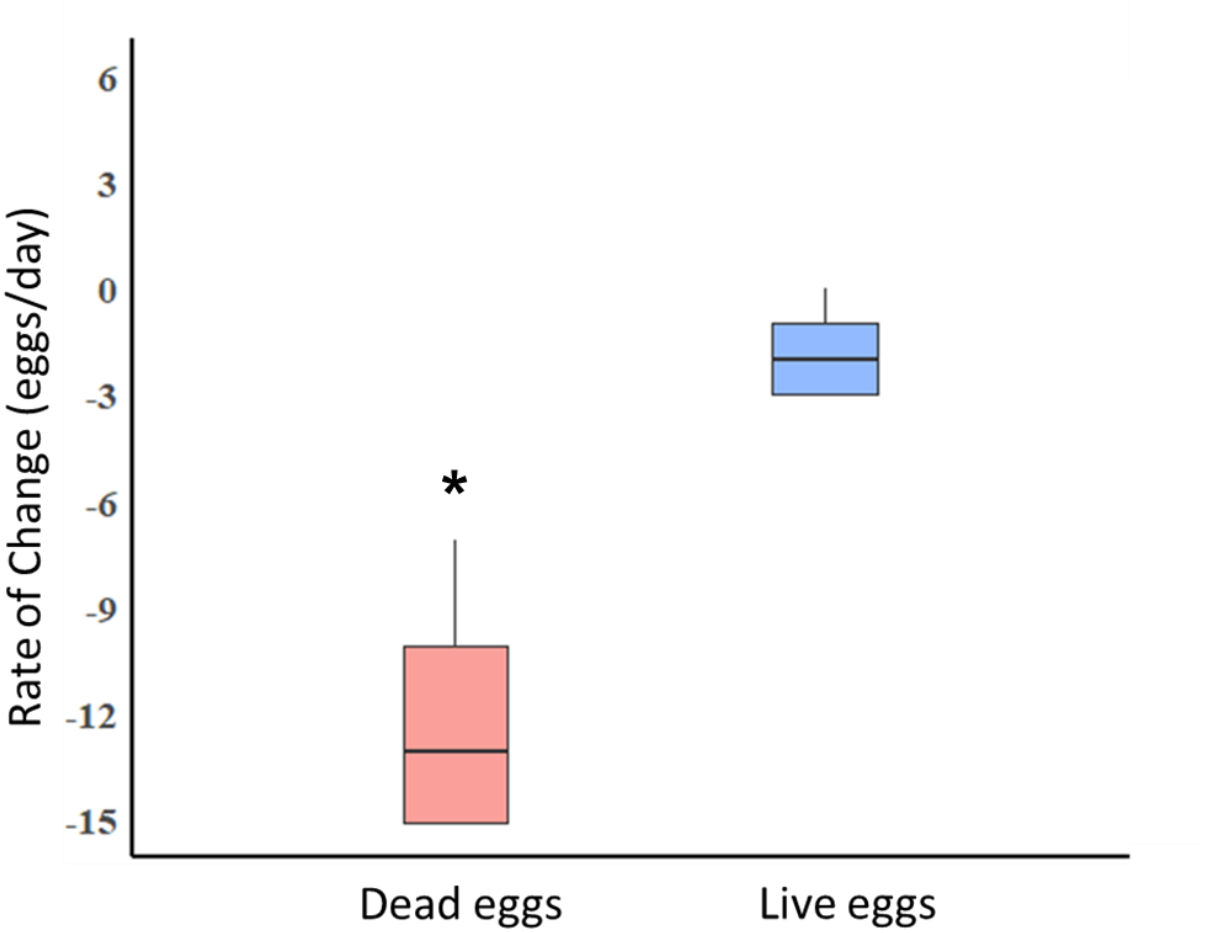

Figure 3.4 Delta Smelt egg consumption rate in the first 24 hours of the experiment (3 DPF to 4 DPF). The asterisk indicates significant difference ($p < 0.05$).

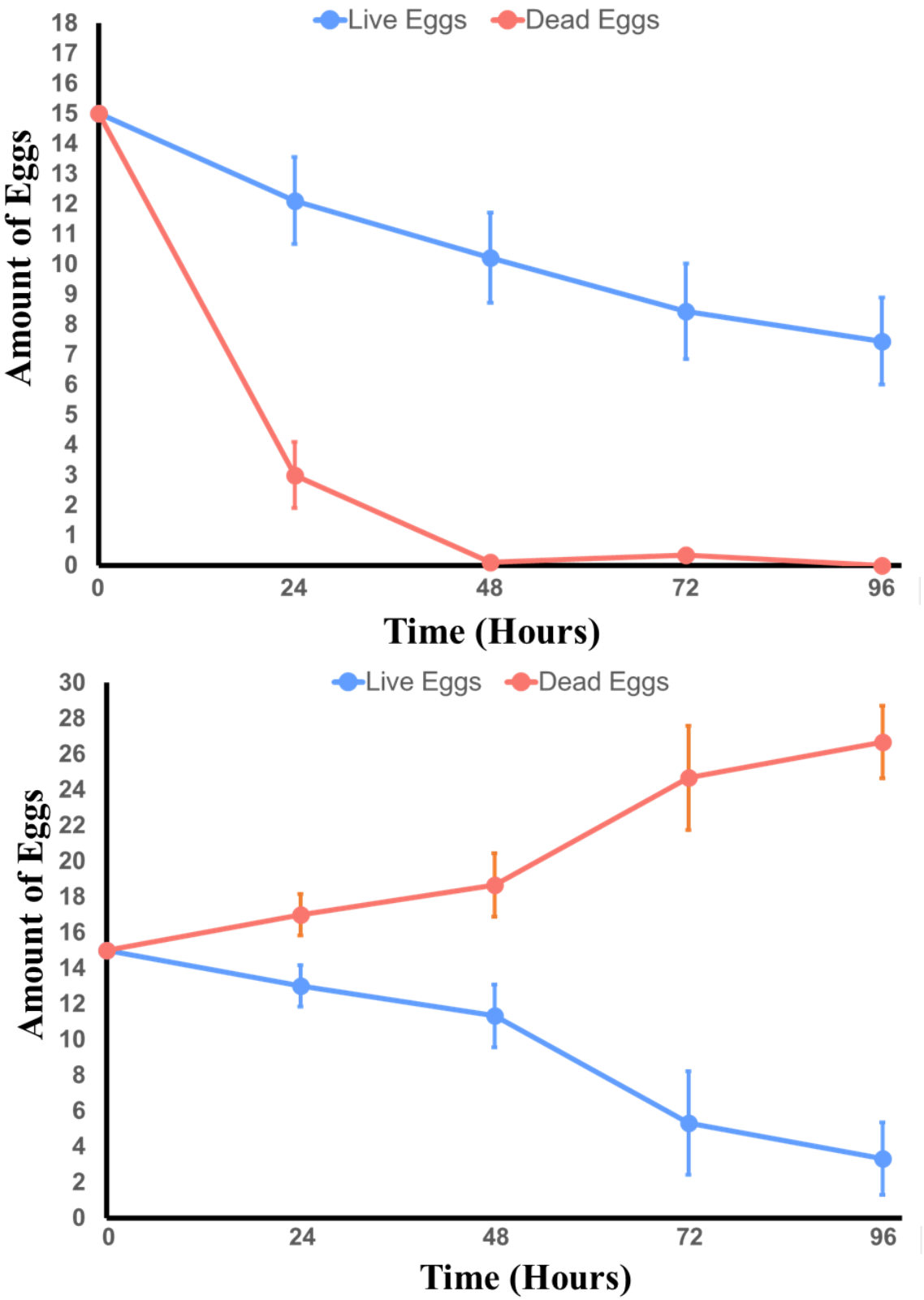

Figure 3.5 The amount of Delta Smelt egg from 3 DPF (0 hours) to 7 DPF (96 hours). The upper part shows the total amount of eggs from 3 DPF to 7 DPF in the experimental groups (n = 9). The lower part shows the total amount of eggs from 3 DPF to 7 DPF in the experimental groups (n = 3).

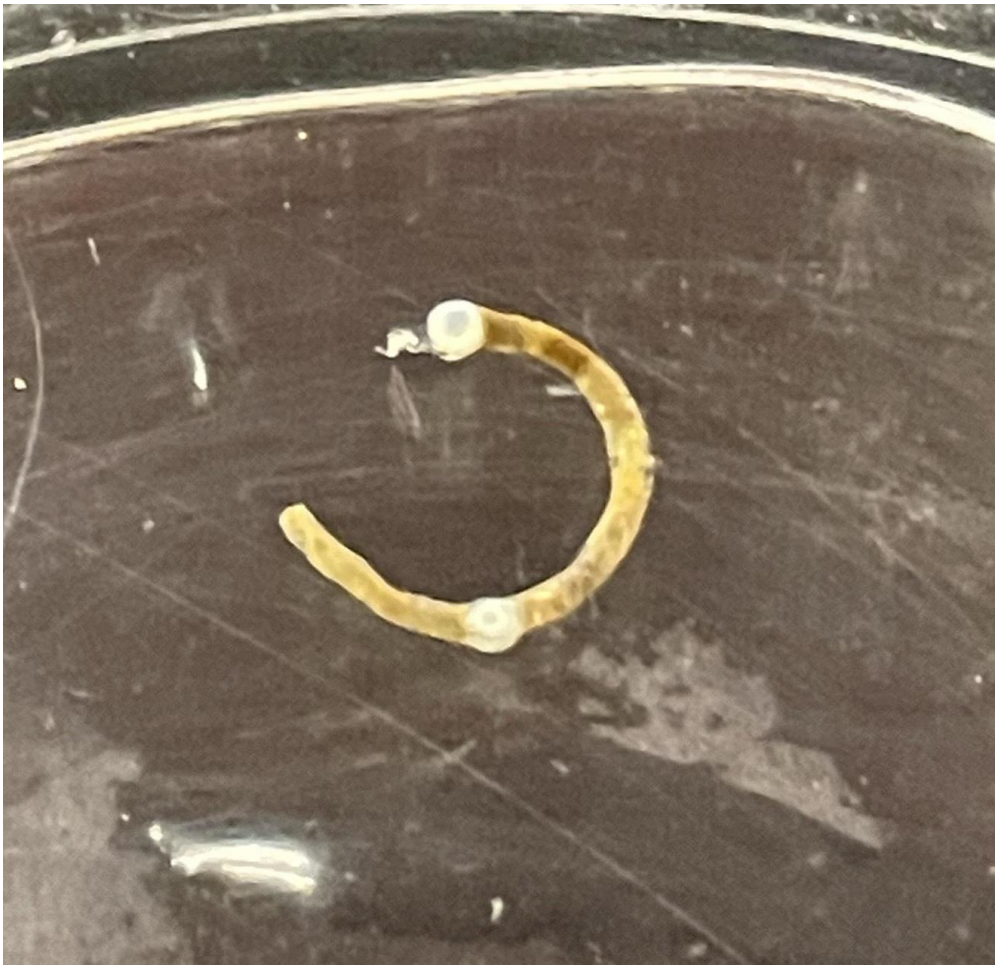

Figure 3.6 Two dead Delta Smelt eggs observed in the feces of a Ramshorn Snail. The eggs presented in the feces remained in sphere shape and were not completely digested by the snails.

3.3.2 SEM Imaging of Delta Smelt Eggs at 3 DPF

To understand the differences between the surfaces of live and dead Delta Smelt eggs, SEM was utilized. This approach provided more details, revealing key distinctions on the surfaces of the eggs at 3 DPF.

Figure 3.7 presents SEM images of both live and dead Delta Smelt eggs. The magnified images captured the complete look of these two types of eggs. Under this magnification of 150x, the surface of the dead egg was observed with apparent fungal growth, while the surface of the live egg was observed relatively clean and smooth. When observing the eggs at 5000x magnification, the images captured closer images of the bacterial growth on the dead egg surface, which was conspicuously more pronounced than the live egg. Moreover, the surface of the dead egg exhibited clear signs of decomposition, presenting as more pores and dents on the surface.

Further magnification, as displayed in Figure 3.8, offered a detailed examination of the surface of the same dead Delta Smelt egg. This 10,000x magnification highlighted the intrusive fungal growth on the dead egg. Most notably, the images highlighted the fungal growth that had penetrated the surface of the dead egg. These SEM images at 3 DPF clearly presented the differences between the surfaces of live and dead eggs. As the dead egg exhibited more decomposition compared to the live one, the prominence of both fungal and bacterial colonization was also evidently more pronounced on it.

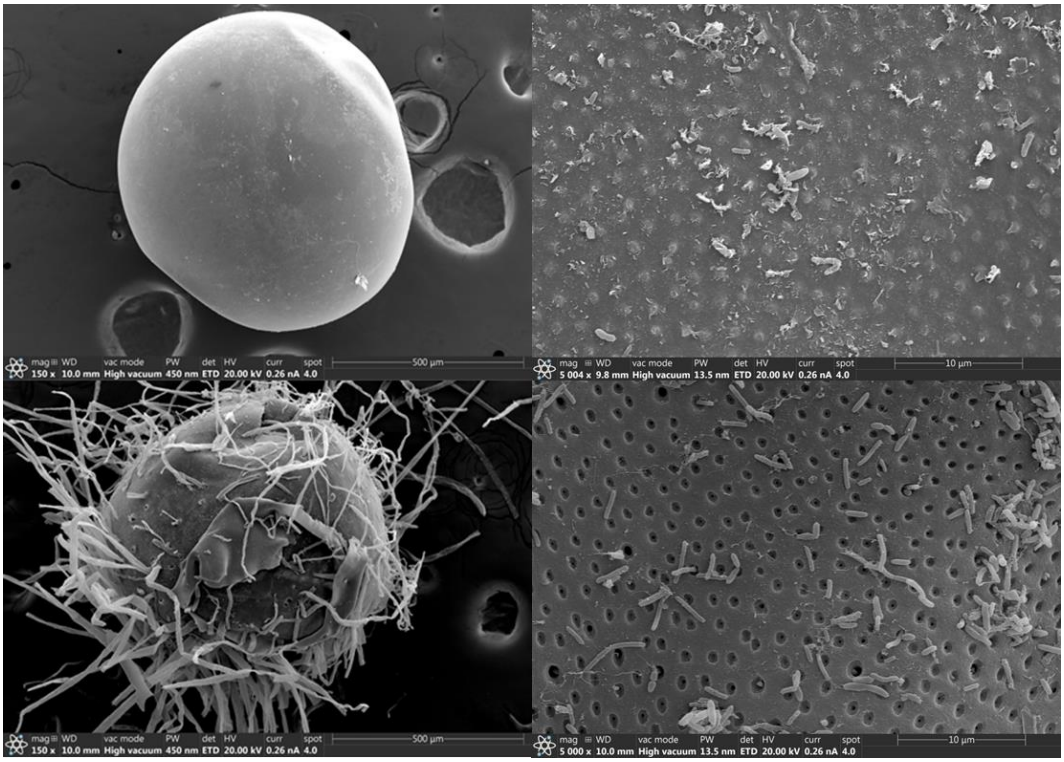

Figure 3.7 SEM images of Delta Smelt eggs at 3 DPF. The top two images show a live egg, while the bottom two images show a dead egg with fungal growth. Images were magnified 150x (two images on the left) and 5000x (two images on the right).

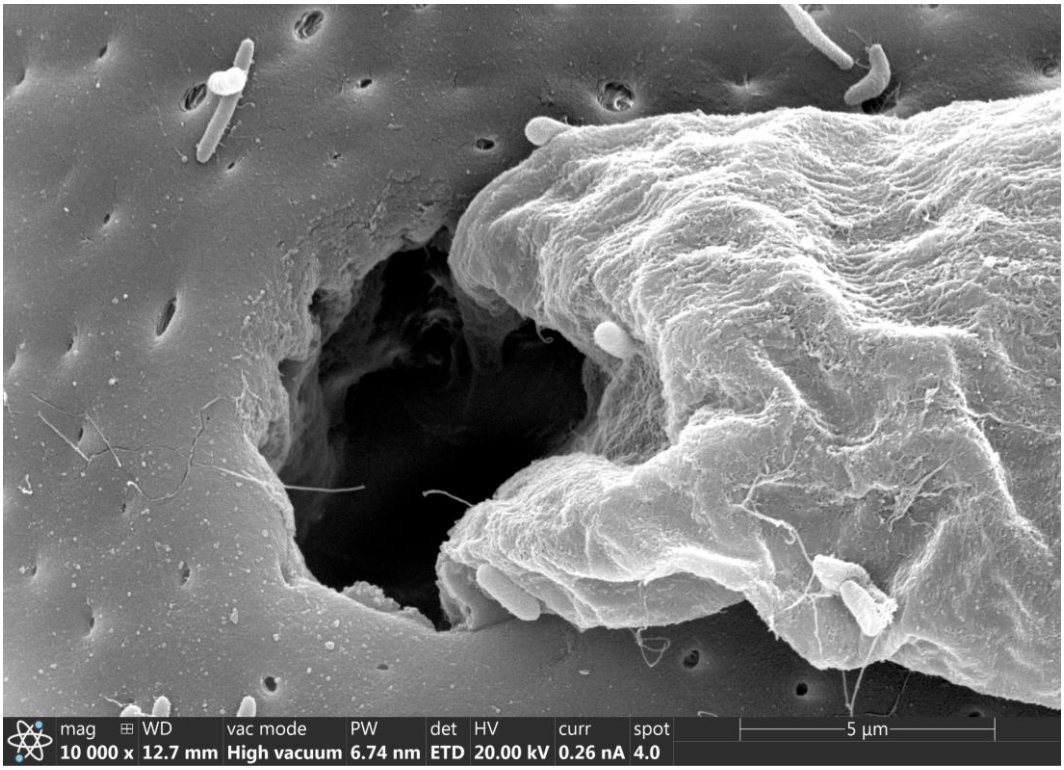

Figure 3.8 An SEM image of a dead Delta Smelt egg at 3 DPF. The image offers a detailed view of the egg, revealing the fungal growth and decomposition on the egg surface. Notably, the image captured the invasive growth of the fungus into the surface of the egg. The image was magnified 10,000x.

3.4 Discussion

3.4.1 Selective Feeding Behavior of Ramshorn Snail

The snails expressed a clear preference for consuming dead Delta Smelt eggs over the live eggs at the beginning of the experiments. The snails consumed most of the dead eggs in the first day (3 to 4 DPF) and by the time the experiment reached 5 DPF, the snails had consumed nearly all the dead Delta Smelt eggs, indicating their strong preference for dead eggs. In contrast, only around 30% of the live eggs had been consumed by the snails by this point. As the study continued, with the majority of dead eggs already consumed, the snails steadily fed on the live eggs. This observed behavior, likely rooted in specific chemosensory cues, suggested that dead eggs may have certain attributes making them more appealing to the snails. During the experiment, it was observed that dead fish eggs emit a relatively strong odor compared to live eggs when submerged in water for several days, despite daily water changes. This distinct odor from the dead fish eggs is likely attributed to fungal and bacterial growth, as well as the decomposition of the eggs (Figure 3.7). Examination under an SEM revealed more pronounced signs of decomposition and bacterial growth on the surface of dead fish eggs. Notably, fungi were seen growing within perforations on the surface of dead fish eggs (Figure 3.8). While it remains uncertain whether this decomposition and fungal growth penetrated the surface of dead fish eggs, the likelihood is substantial. The decomposition and fungal growth could lead to the release of substances from these dead eggs, which in turn might attract the snails. Such factors could explain the preference of these snails for dead Delta Smelt eggs over the live ones.

3.4.2 Implications for Fish Egg Separation

The behavior exhibited by the Ramshorn Snails in this study presented valuable insights for hatchery operations. Their evident preference towards dead eggs suggested a

potential eco-friendly approach to remove dead eggs from the live ones. As highlighted in earlier chapters, traditional methods of fish egg separation require significant labor. Incorporating Ramshorn Snails as natural fish egg separators could provide continuous dead fish egg removal with minimal oversight. The benefits of this approach include reduced labor requirements and easy integration into existing separation procedures without major modifications, thereby minimizing the introduction of untested steps or methods. Furthermore, the FCCL has experience with Ramshorn Snails, eliminating the need to introduce a new species and allowing for the expansion of current Ramshorn Snail cultivation if necessary. While this study focused on the eggs of Delta Smelt, the mechanism by how Ramshorn Snails detect food suggested that this method might also be suitable for eggs of other fish species, such as Longfin Smelt. However, this applicability across species remains unverified, requiring further research to confirm the broader applicability of Ramshorn Snails in fish egg separation. Another consideration when employing snails for fish egg separation relates to the size of the eggs. Given that Ramshorn Snails are typically smaller than 20 mm, their application might be less practical for fish species with larger egg size, such as Salmonids (egg size around 5.5-7 mm in diameter [Reid & Chaput, 2012].). The snails could potentially face difficulties in ingesting such sizable eggs. Future studies might focus on the application of other larger aquatic gastropods to understand the applicable across different snail species.

Finally, understanding the specific cues or attributes that make dead eggs more attractive to these snails could open up avenues for developing other natural or chemical methods for aquaculture practices, including the egg separation. As the aquaculture industry continues to seek efficient, sustainable, and environmentally friendly methods, such natural solutions could play an important role in shaping the future of fish cultivation practices.

3.4.4 Concerns of Ramshorn Snails in Fish Egg Separation

While this study has highlighted the preference of Ramshorn Snails for dead eggs, there are some concerns associated with this egg separation method. During the process of utilizing Ramshorn Snails for Delta Smelt egg separation, the snails constantly produced feces during the incubation. The presence of the feces during the incubation process requires more steps for the staff to separate them from the eggs. Additionally, the presence of fish eggs in the feces also suggested that the snails might not have fully digested the eggs. The exact reason for this partial digestion remains unclear. One potential explanation could be the composition of the outer chorion layer of the fish eggs, which is majorly made of proteins and glycoproteins (Cotelli et al., 1988). The structure of the chorion comprises a dense inner layer, known as the zona radiata interna, and a finer external layer termed the zona radiata externa (ZRE) (Huysentruyt and Adriaens, 2005). Notably, while non-adhesive eggs typically possess a thinner and simpler structured ZRE, adhesive eggs, such as those of the Delta Smelt, are encased in a thicker and more complicated structured ZRE (Riehl and Patzner, 1998; Rizzo et al., 1998). This ZRE structure might be the reason for some snails not being able to digest the eggs completely. Some proteolytic enzymes might be able to solve this problem, as it removes some parts of the chorion layer of fish eggs (Bian et al., 2022) and might make the eggs more digestible for the snails. Future research might focus on the efficient method of removing these feces from the incubation process and the digestive system of the Ramshorn Snails to identify the reasons for this indigestion.

Another concern in utilizing Ramshorn Snails for fish egg separation is from their inherent nature as living organisms. The behavior of living animals, including the Ramshorn Snails, can be unpredictable and challenging to control comprehensively. Their presence during the hatching process introduces other variables. For instance, their feces, mucus, and metabolic by-products could potentially alter water quality, introducing

additional variables to the fish egg production process. Given these complexities, it is important to conduct further experiments to seek for better methods to minimize the impacts from these potential variables, especially for large-scale fish egg production. The implications of having these snails in the systems also require more in-depth exploration and observation.

3.5 Conclusion

The study in this chapter highlights the value of Ramshorn Snails as a potential solution for fish egg separation. Traditionally, the separation of dead fish eggs at the hatchery is a labor and time intensive task, all relying on manual processes. The Ramshorn Snails, however, offer an ecological approach, leveraging their natural preference for consuming dead organisms and detritus. Our observations highlighted a pronounced dietary preference in the snails towards dead Delta Smelt eggs during the initial days of the experiment. Such behavior, rooted in chemosensory cues, suggests that dead eggs, with their distinct attributes and odors, appeal more to these gastropods.

Further details were gathered through the SEM imaging, which revealed that dead eggs exhibited notable signs of decomposition and fungal growth, providing further clues to their appeal to the snails. Yet, when applying this method to larger hatchery operations, complications might arise. While the snails efficiently consumed dead eggs, they also ingested live ones. Furthermore, the presence of snails during the incubation period might alter water quality due to their metabolic by-products, feces, and mucus. While the approach using Ramshorn Snails presented a novel solution, its implementation, especially in larger-scale operations, necessitates more thorough evaluation and consideration.

Lastly, it is also essential to consider the broader conservation landscape. Given the endangered status of the Delta Smelt, hatcheries like the FCCL play a critical role in their

conservation. As such, any new methodology should not only enhance operational efficiency but should also resonate with the natural conditions of the species and well-being. In conclusion, while Ramshorn Snails offer a promising avenue for fish egg separation, the approach requires additional refinement and studies, especially considering the larger-scale and cross-species applications.

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CHAPTER 4

COMPARING DIFFERENT EGG ADHESIVENESS REMOVAL METHODS

4.1 Introduction

4.1.1 The Adhesiveness of Fish Eggs

Teleost fish eggs are encased in a protein-rich layer known as the chorion. This protective layer is bifurcated into two distinct sections, the denser inner section, the zona radiata interna, and the more delicate outer section, the zona radiata externa (ZRE, Huysentruyt and Adriaens, 2005). While a majority of teleost species exhibit this chorionic structure, its specific morphology and biochemical composition can differ across species, leading to variations in egg adhesiveness (Arukwe and Goksøyr, 2003; Riehl and Patzner, 1998). Current studies suggest that the shape of the chorion and its biochemical properties play a critical role in egg adhesiveness. In certain species in the order of *Characiformes*, the structure of the ZRE plays a critical role in determining adhesiveness. Furthermore, compounds like glycoproteins and mucopolysaccharides are thought to be key players in this adhesive process (Cherr and Clark Jr, 1982; Debus et al., 2008). Fish eggs that do not adhere to surfaces typically have a ZRE that is simple and thin. On the other hand, eggs that are adhesive possess a ZRE that is both thicker and more complicatedly structured (Riehl and Patzner, 1998; Rizzo et al., 1998). This adhesiveness of fish eggs is not just a random trait. Instead, it is an evolutionary adaptation. By adhering to surfaces, fish eggs are less likely to be swept away by environmental elements like water currents, ensuring they remain in favorable habitats (Mansour et al., 2009a; Murray et al., 2013). Additionally, when eggs are anchored in specific territories, they are more likely to receive protection from potential parent fish, boosting their chances of survival (Mansour et al., 2009b).

However, this natural adhesiveness is not without its challenges, especially in controlled breeding and hatchery environments. The adhesiveness complicates the egg collection process, raises the risk of infections, and makes it tough to distinguish between healthy and non-viable eggs (Siddique et al., 2016; Grant et al., 2016). To overcome the challenges posed by fish egg adhesiveness, both physical and chemical solutions have been explored. While physical methods involve using abrasive solutions, chemical methods utilize specific compounds to reduce the egg adhesiveness (Chebanov and Galich, 2011; Monaco and Doroshov, 1983).

4.1.2 Egg Adhesiveness in Delta Smelt and Longfin Smelt

For Delta Smelt (*Hypomesus transpacificus*) and Longfin Smelt (*Spirinchus thaleichthys*), the adhesiveness on eggs also presents as an adaptation to the higher water flow velocity of their natural habitats (Lindberg et al., 2020). Generally, the adhesiveness of fish eggs is a natural characteristic that helps in the survival of the species by allowing the eggs to attach to substrates in their environment, thereby reducing the risk of predation and dispersion (Baskerville-Bridges et al., 2005). However, in the case of aquaculture practices and conservation projects, this egg adhesiveness can pose challenges, particularly in the handling, sorting, and incubation of the fish eggs. Therefore, the removal of egg adhesiveness is a critical step in the aquaculture and conservation of many fish species (Siddique et al., 2016), including Delta Smelt and Longfin Smelt. In this chapter, four different methods are explored and compared for the removal of the adhesiveness of Delta Smelt eggs. These methods include the standard bentonite clay treatment currently used at the UC Davis Fish Conservation and Culture Lab (FCCL, Byron, CA), as well as three alternative treatments: sodium hypochlorite treatment, proteolytic enzyme treatment, and tannic acid treatment. The enzyme treatment and tannic acid treatment methods were

inspired by previous studies conducted on Pikeperch (*Sander lucioperca*) eggs (Demska-Zakęś et al., 2005; Kristan et al., 2016), while the sodium hypochlorite treatment was developed based on their previous application on Delta Smelt eggs at the FCCL (Tsai et al., 2021).

The objective of this chapter is to assess the efficacy of these four methods in removing the adhesiveness and to compare their impacts on the eggs. The findings of this study will contribute to the optimization of fish egg production and incubation processes in the conservation projects of Delta Smelt and Longfin Smelt. Finally, the results are expected to be used as an aid for the alternative fish eggs sorting methods developed in Chapter 2 and 3. Furthermore, the insights gained could potentially be applied to aquaculture practices or conservation programs for other species such as Eulachon (*Thaleichthys pacificus*), thereby broadening the scope of this research.

4.2 Methods and Materials

Following previous studies on different species, each of the methods mentioned in 4.1 involves a mechanical detachment process, where the eggs are gently rubbed off the surface of the bowl with fingers. This is followed by a stirring process in the solution, except for the bentonite clay treatment, which only involves the mechanical detachment process, following the standard operating procedure for the bentonite clay treatment at the FCCL (Lindberg et al., 2013). After each adhesiveness removal treatment, the eggs were rinsed twice with Delta Smelt incubation system water (16.5°C and 0 ppt salinity, Lindberg et al., 2013) to ensure the complete removal of any remaining solution.

4.2.1 Fish Eggs Production and Incubation Pre-treatment

In this chapter, the experiments were conducted exclusively with Delta Smelt eggs. For the fish eggs production in this study, we employed 12 Delta Smelt crosses, each involving a pairing of one female with one male. Mature males and females were selected based on tactile cues, which the maturity of a male or female fish was determined by gently applying pressure on their abdomen with fingers to check for the presence of milt and eggs, respectively. When fish are mature and ready to reproduce, milt and eggs can be easily expressed from their bodies with minimal pressure applied to their abdomen (Ellison et al., 2023). For each group, eggs were first stripped from a single female Delta Smelt and placed in a dry bowl. Subsequently, milt was stripped from a male fish and immediately placed directly onto the eggs. The eggs and milt were then mixed together by gently stirring with a plastic stick. The eggs were then distributed into 12 small bowls for each group separately, with each bowl containing 80 to 100 eggs. 60 mL of Delta Smelt incubation system water was then added to each small bowl to activate the fertilization process. Upon contact with the water, the eggs became adhesive and attached to the surface of the bowl. After a period of one minute, the water in each bowl was replaced once with the Delta Smelt incubation system water. These 12 bowls containing adhesive fish eggs were then placed in the sump of a Delta Smelt incubation system (Figure 3.3) and maintained at a constant temperature of 16.5°C for a three-day period. During incubation, the water in the bowls was replaced every 24 hours, and a one-minute disinfection treatment with 14.5 mL of Pond Rid-Ich Plus per liter of freshwater (Kordon, LLC, Hayward, California, USA: active ingredients: 4.26% formaldehyde and 0.038% zinc-free chloride salt of malachite green) was applied to the eggs to minimize fungal growth, as suggested by Baskerville-Bridges et al. (2005). At the time of three days post-fertilization (DPF), the number of the eggs in each bowl was counted, and the eggs were then ready for the adhesiveness removal experiments.

4.2.2 Experiment Design

In this study, three alternative methods and one standard bentonite clay treatment for removing egg adhesiveness were employed (Table 4.1). The standard bentonite clay treatment (Lindberg et al., 2013) was utilized as the control group for three different treatment methods. Three alternative methods included proteolytic enzyme treatment, tannic acid treatment, and sodium hypochlorite treatment. For the tannic acid and sodium hypochlorite treatments, three different solution concentrations (500 mg/ L, 1000 mg/L, and 1500 mg/L for the former and 0.075%, 0.135%, and 0.210% by volume for the latter) were tested as suggested by previous studies (Demska-Zakęś et al., 2005; Tsai et al., 2021). Meanwhile, six different solution concentrations (0.5%, 1%, 1.5%, 2%, 3%, and 5%) were examined for the proteolytic enzyme treatment. The concentrations for the proteolytic enzyme treatment were chosen based on the previous study on Pikeperch (Kristan et al., 2016). In the study, the authors suggested that the effective concentration of the enzyme solution varies between different species. Therefore, a broader range of the concentrations was selected to ensure the optimal condition was included. For each different treatment method and concentration, fish eggs from 3 separate crosses of Delta Smelt were tested independently. Each cross and concentration was replicated 3 times, resulting in a total of 9 tests for each different treatment concentration value (i.e., 3 crosses of Delta Smelt × 3 replicates = 9 tests for each different treatment concentration value).

Table 4.1 Different egg adhesiveness removal treatments. Four different treatments including three alternative methods with various concentrations and one standard bentonite clay treatment.

Treatment	Concentration	Treatment time and detachment method
Bentonite Clay	16 g /L	1 minute, mechanical detachment
Sodium hypochlorite	0.075% (v/v)	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Sodium hypochlorite	0.135% (v/v)	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Sodium hypochlorite	0.210% (v/v)	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Alcalase enzyme	0.5% (v/v)	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Alcalase enzyme	1% (v/v)	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Alcalase enzyme	1.5% (v/v)	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Alcalase enzyme	2% (v/v)	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Alcalase enzyme	3% (v/v)	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Alcalase enzyme	5% (v/v)	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Tannic acid	500 mg/L	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Tannic acid	1000 mg/L	2 minutes, 1 minute mechanical detachment + 1 minute stirring
Tannic acid	1500 mg/L	2 minutes, 1 minute mechanical detachment + 1 minute stirring

4.2.3 Standard Bentonite Treatment

The standard fish egg adhesiveness removal method employed at the FCCL involves the use of a bentonite clay mixture (Sigma-Aldrich, Inc., Saint Louis, Missouri, USA). This

mixture was prepared by blending 16 grams of bentonite clay powder with 1 liter of hatching system water using a commercial countertop blender, ensuring a thorough mix. In this study, distilled water was used to prepare the clay mixture to ensure consistency across various treatments, rather than using water from the hatching systems. The standard bentonite clay treatment was conducted by adding a 100 mL of the clay mixture to a bowl containing adhesive fish eggs. The water and non-adhesive eggs were carefully poured out from the bowl before adding the clay mixture. At the time of adding the clay mixture, all of the eggs were attached to the surface of the bowl. To remove the adhesiveness and detach the eggs from the bowl surface, a one-minute mechanical detachment process was performed by gently rubbing the eggs off the surface with fingers. This process, unlike the other three alternative methods, was completed without the need for additional stirring. Following the one-minute treatment, the fish eggs, along with the clay mixture, were poured into a fine mesh (500 micron) strainer in order to separate the eggs from the clay slurry. The strainer was then rinsed twice with Delta Smelt incubation system water to ensure a complete removal of the rest of the clay mixture. The eggs were then transferred back to the bowls for the incubation.

4.2.4 Alternative Fish Eggs Adhesiveness Removal Treatment Methods

For alternative fish eggs adhesiveness removal treatments, three different treatment methods were selected: proteolytic enzyme treatment, tannic acid treatment, and sodium hypochlorite treatment. Similar to the bentonite method, a 100 mL of each solution was added to bowls containing adhesive fish eggs, with the water in the bowl being removed prior to the addition of the solutions. For all three alternative methods, the fish eggs were first mechanically detached from the surface of the bowl by gently rubbing them off with fingers for 1 minute, within the solution. This was followed by an additional 1 minute of

stirring with a plastic stick in the solution within the bowl. The method contrasts with the standard bentonite clay treatment, which only involved a one-minute mechanical detachment with fingers and did not include the additional minute of stirring (Table 4.1). Following the same procedure as the standard bentonite clay treatment, after each alternative treatment, the eggs were poured into a fine mesh (500 micron) strainer and rinsed twice with incubation system water twice to ensure the complete removal of any remaining chemical solution. In this study, all three alternative methods were designed to be similar to the standard bentonite treatment, along with a hatching rate determination process.

4.2.4.1 Sodium hypochlorite treatment

Commercial liquid bleach (8.25% sodium hypochlorite, Clorox, USA) was purchased and utilized. Three distinct concentrations of sodium hypochlorite solution were prepared for the experiments by diluting the liquid bleach with distilled water. The three diluted concentrations were: 0.075%, 0.135%, and 0.210% sodium hypochlorite by volume. Three bowls of Delta Smelt eggs were tested for each concentration. Sodium hypochlorite has been verified in a previous study of being able to remove the adhesiveness of Delta Smelt eggs (Tsai et al., 2021) at the concentration of 0.13% (v/v). However, the hatching rate of the eggs after the treatment was not tested. The potential impacts to the hatching rate from the treatment remained unknown.

4.2.4.2 Proteolytic enzyme treatment

A commercial proteolytic enzyme product, the Alcalase enzyme (*Bacillus licheniformis*, MilliporeSigma, USA) was chosen (Kristan et al., 2016). Based on the same study of Kristan et al. in 2016, six distinct concentrations of the Alcalase enzyme solution

were prepared for the experiment: 0.5%, 1%, 1.5%, 2%, 3%, and 5% (2.9718 U/mL) of enzyme by volume. Same as the other treatment methods, three bowls of Delta Smelt eggs were tested for each concentration.

4.2.4.3 Tannic acid treatment

Three distinct concentrations of tannic acid solution were prepared for the experiments: 500, 1000, and 1500 mg of tannic acid powder ($C_{76}H_{52}O_{46}$, Thermo Scientific, USA) thoroughly mixed with 1 liter of distilled water. The concentrations of the solutions were chosen based on the previous research conducted on the eggs of Pikeperch (Demska-Zakęś et al., 2005). Same as the other treatment methods, three bowls of Delta Smelt eggs were tested for each concentration.

4.2.5 Scanning Electron Microscope Imaging

In order to understand what happened to the surface of the fish eggs after different adhesiveness removal treatments, the eggs were examined by utilizing a scanning electron microscope (SEM, Quattro S Environmental Scanning Electron Microscope, ThermoFisher, USA). Ten different kinds of eggs were examined at 3 DPF, and all the eggs were obtained from the same cross of Delta Smelt (Table 4.2). Images were captured under an accelerating voltage of 20 kv.

Prior to the SEM imaging, the egg samples underwent a series of preparation steps. The fixation, dehydration and gold coating process for the egg samples were conducted by the UC Davis Biological Electron Microscopy Facility. The eggs were initially fixed using a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. After fixation, the samples underwent dehydration using ethanol to remove any remaining moisture. This was followed by critical point drying, a process employed to preserve the

structural integrity of the samples. Finally, before the SEM imaging process, the dried samples were sputter-coated with gold.

Table 4.2 Ten different kinds of Delta Smelt eggs examined by the SEM. Mechanical Detachment was performed by rubbing the eggs off from the surface of the bowl using fingers. Other treatment methods followed the procedures described in Section 4.2.4.

Egg Type	Treatment	Treatment Time
Live eggs	Mechanically detached	N/A
Live eggs	Bentonite Clay	1 minutes
Dead eggs	Bentonite Clay	1 minutes
Live eggs	2% Alcalase enzyme	2 minutes
Live eggs	5% Alcalase enzyme	2 minutes
Live eggs	500 mg/L tannic acid	2 minutes
Live eggs	1500 mg/L tannic acid	2 minutes
Live eggs	0.075% sodium hypochlorite	2 minutes
Live eggs	0.2% sodium hypochlorite	2 minutes
Dead eggs with fungal growth	Mechanically detached	N/A

4.2.6 Adhesiveness Removal Effectiveness

The effectiveness of the four different adhesiveness removal treatment methods was evaluated. 50 eggs from each group were collected after the adhesiveness removal treatment using a plastic pipette (Fisherbrand™ Standard Disposable Transfer Pipettes, capacity: 7.7 mL; Molecular Bio Products Inc., USA). Of these 50 eggs, those that were successfully transferred to another bowl were considered to have had their adhesiveness

successfully removed. Conversely, eggs that were still showing stickiness and remained attached to the inner surface of the pipette and could not be dislodged by water flow were classified to still possess their adhesiveness. The adhesiveness removal effectiveness was calculated for each group and was categorized into four distinct adhesiveness removal levels, labeled as levels 0 through 3. Level 0 indicated that the adhesiveness was not removed at all or barely removed, with all or most of the eggs remaining adhesive and unable to be dislodged from the plastic pipette. Conversely, level 3 represented a complete removal or near complete removal of adhesiveness, with all or most of the eggs being easily dislodged from the plastic pipette. The levels in between (1 and 2) represented varying degrees of adhesiveness removal effectiveness, with level 1 indicating poor adhesiveness removal effectiveness with the majority of the eggs still remaining adhesive and level 2 indicating moderate adhesiveness removal effectiveness, with some eggs still remaining adhesive. This classification system was employed based on the observation from the egg adhesiveness removal processes. During the experiments, some eggs were observed to possess partial adhesiveness, which the eggs adhered to the plastic pipette sometimes and were able to be dislodged sometimes as well. This classification system was employed instead of calculating the adhesiveness removal rate directly in percentage, which provided a clearer measure to assess the effectiveness of the different adhesiveness removal methods.

4.2.7 Hatching Rate

To evaluate the potential impacts of different adhesiveness removal treatments on the viability of Delta Smelt eggs, a hatching rate determination process was implemented. This process was designed to provide a quantitative measure of the safety for applying each treatment method on the fish eggs. For each treatment method and concentration, a sample of 20 fertilized eggs was randomly selected from each group for the test. This process was

replicated nine times for each concentration value (i.e., 3 different Delta Smelt crosses × 3 replicates = 9 tests for each concentration value), ensuring a robust dataset for analysis.

Following the adhesiveness removal treatments, the selected eggs were transferred to a new clean bowl with 150 mL of Delta Smelt incubation system water and incubated in a water bath maintained at a constant temperature of 16.5°C. This temperature was chosen to match the optimal incubation conditions for Delta Smelt eggs, as established in previous studies (Lindberg et al., 2013). The eggs were incubated for a period of 7 days, during which the water in the bowls was changed daily by using the water from the same incubation system. This water change process was the only procedure performed during the incubation period, minimizing any potential external influences on the hatching process. Along with each water change, any dead eggs were carefully removed and documented once daily. This allowed for the tracking of egg viability throughout the incubation period and provided a clear measure of the impact of each treatment method on egg survival and excluded the potential impacts from the dead eggs existing in the bowl. At the end of the seven-day incubation period, the hatching rate was calculated for each group at 10 DPF. This was expressed as a percentage, providing a quantifiable measure of the influences from different treatment methods to the egg hatching rates.

4.2.8 Statistical Analysis

All statistical analyses were conducted using the R programming environment (R Core Team, 2023) version 4.3.1. The employed libraries for facilitating the analyses included: data processing and manipulation libraries: dplyr (Wickham et al., 2021), dunn.test (Dinno, 2017), PMCMRplus (Pohlert, 2018), and car (Fox & Weisberg, 2019), data visualization library: ggplot2 (Wickham, 2016). and other utilities: extrafont (Chang, 2014).

4.2.8.1 Adhesiveness Removal Effectiveness across Treatment Methods

In order to understand the adhesiveness removal effectiveness across different treatment methods, the adhesiveness removal grades for each method were documented and compared. Observing identical grades across all replicates for every treatment method suggested a non-normal distribution of the data. Thus, the non-parametric Kruskal-Wallis test was employed to determine if statistically significant differences in adhesiveness removal grades were present among the different methods. The test revealed a highly significant result ($p = 2.2\times10^{-16}$), suggesting significant differences exist in median values of adhesiveness removal grades across the treatments.

Following the Kruskal-Wallis test, a post-hoc analysis using Dunn's test was utilized, paired with a Bonferroni correction to adjust for multiple comparisons. The objective was to identify specific treatment methods that displayed significant deviations in adhesiveness removal grades compared to the traditional bentonite clay treatment.

4.2.8.2 Hatching Rate in Relation to Treatment Methods

Prior to performing inferential statistics, two main assumptions of normality and homogeneity of variances were tested. The residuals of a linear model, constructed with hatching rate as the dependent variable and treatment as the predictor, were subjected to the Shapiro-Wilk test. The test yielded a p-value of 0.04445, which fell below the significance threshold of 0.05, indicating a deviation from normality. Further, the Levene's test revealed unequal variances across treatments, as evidenced by a p-value of 1.809×10^{-5}. Given the violations in the assumptions of normality and homogeneity of variances, the non-parametric Kruskal-Wallis rank sum test was applied to discern differences in the hatching rates among treatments. Following the Kruskal-Wallis test, a post-hoc analysis utilizing Dunn's test was conducted, paired with a Bonferroni correction to adjust for

multiple testing. This aimed to determine the specific treatment methods that significantly differed in hatching rates from the traditional bentonite clay treatment.

4.3 Results

4.3.1 Adhesiveness Removal Effectiveness

Due to the observed non-normal distribution in the adhesiveness removal data, the non-parametric Kruskal-Wallis test was deployed to determine differences in adhesiveness removal grades between different treatments. The test yielded a highly significant p-value of 2.2×10^{-16}, indicating notable differences in adhesiveness removal grades across the treatments. Following the Kruskal-Wallis test, post-hoc pairwise comparisons were executed using Dunn's test with a Bonferroni correction to account for multiple testing. Several pairwise comparisons were made among the treatments. For clarity in this section, attention is specifically given to comparisons involving the traditional bentonite clay treatment against other methods. A closer examination of the results indicates that the certain Alcalase concentrations differed in adhesiveness removal grade when compared to traditional bentonite clay treatment. Specifically, Alcalase concentrations of 0.5% (p-value = 7.36×10^{-10}), 1% (p-value = 7.36×10^{-10}), 1.5% (p-value = 6.10×10^{-7}), 2% (p-value = 1.69×10^{-4}), and 3% (p-value = 1.69×10^{-4}) demonstrated significant differences in adhesiveness removal grade when compared to the traditional bentonite clay treatment (Figure 4.1).

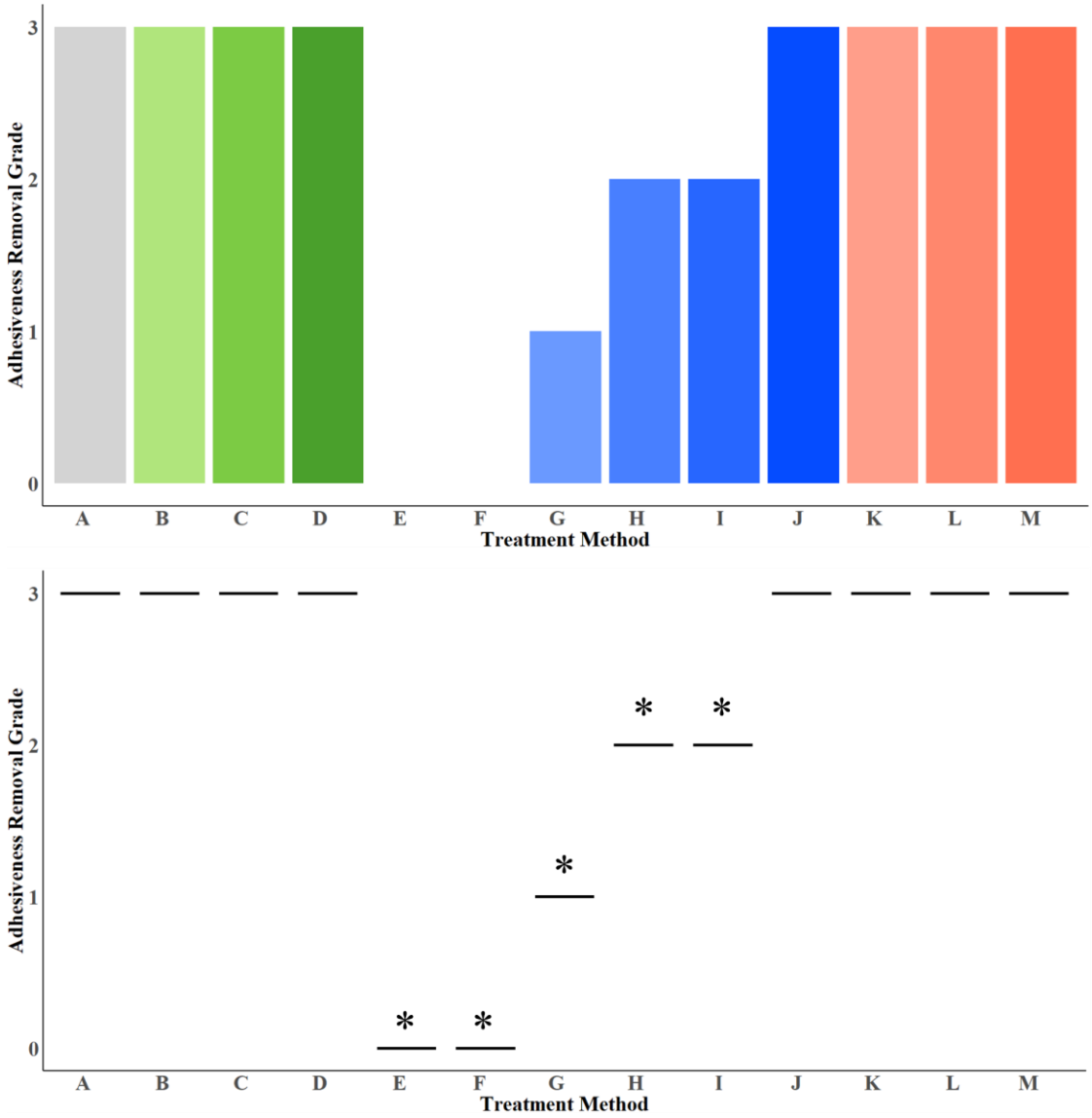

Figure 4.1 Adhesiveness removal grading for Delta Smelt eggs across different treatment methods. The effectiveness is graded from 0 (poor removal) to 3 (complete or near complete removal). The top graph showed the average grade for different methods, while the bottom graph showed the median grade for different methods. The asterisks indicate significant difference ($p < 0.05$) when comparing the adhesiveness removal grade to the traditional bentonite clay treatment. The letter denotes the different egg treatment method: A for bentonite clay, B for 0.075% sodium hypochlorite, C for 0.135% sodium hypochlorite, D for 0.21% sodium hypochlorite, E for 0.5% Alcalase, F for 1% Alcalase, G for 1.5% Alcalase, H for 2% Alcalase, I for 3% Alcalase, J for 5% Alcalase, K for 500 mg/L tannic acid, L for 1000 mg/L tannic acid, M for 1500 mg/L tannic acid.

4.3.2 Hatching Rate in Relation to Treatment Methods

Given the violations in the assumptions of normality and homogeneity of variances, the non-parametric Kruskal-Wallis test was conducted to determine differences in hatching

rates across various treatments. The test yielded a highly significant p-value of 1.599×10^{-12}, which indicates significant differences in hatching rates among the treatments. Subsequent to the Kruskal-Wallis test, post-hoc pairwise comparisons were conducted using Dunn's test with a Bonferroni correction for multiple comparisons. A multitude of pairwise comparisons were made among the treatments. The results revealed comprehensive comparisons of the hatching rate between different treatment methods. In this section, we specifically filtered out the comparisons between traditional bentonite clay treatment and other alternative treatment methods. When compared to the hatching rates yielded from the traditional bentonite clay treatment, all three sodium hypochlorite treatments exhibited significantly lower hatching rates. Given that the hatching rates for all three distinct sodium hypochlorite treatment concentrations were 0% with no exceptions, the p-values generated from the three sodium hypochlorite concentrations of 0.075%, 0.135%, and 0.21% were identically valued at 4.624×10^{-4}. For other treatments, no significant differences were observed, as the adjusted p-values were greater than 0.05, suggesting that these treatments did not have a statistically significant impact on the hatching rates relative to each other.

In summary, the results suggested that the hatching rates were not significantly different for alternative treatment methods when compared to the traditional bentonite clay treatment except for the sodium hypochlorite treatments. Within the testing concentration range of the sodium hypochlorite treatments, all the eggs were killed or became unable to hatch after being treated with sodium hypochlorite solutions. Other treatments, on the other hand, showed similar hatching rates compared to the traditional bentonite clay treatment.

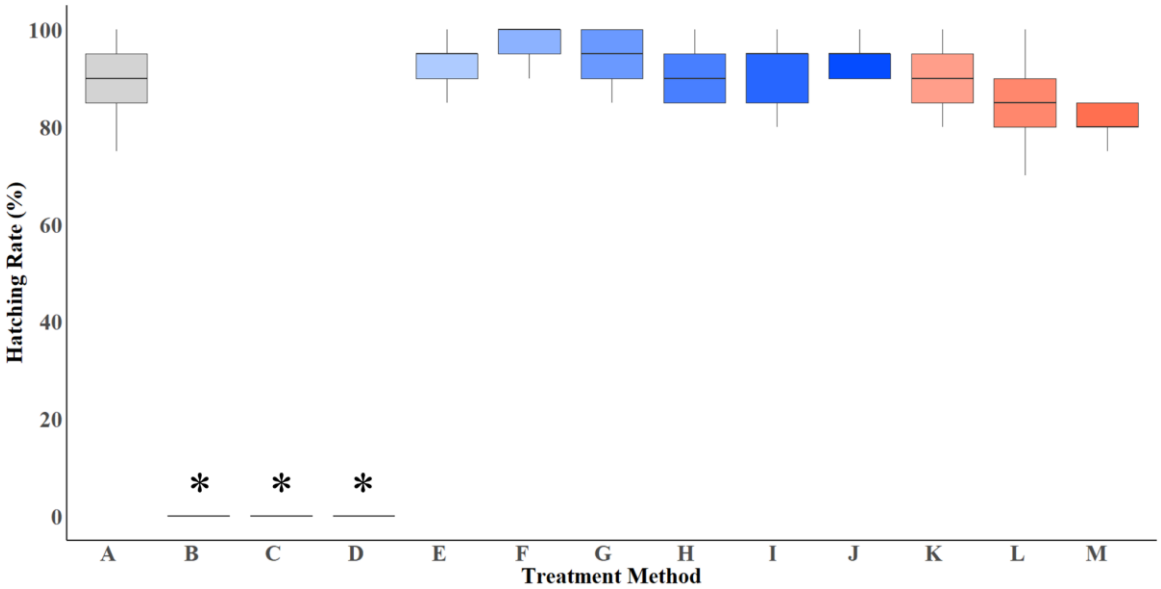

Figure 4.2 Delta Smelt egg hatching rates under different egg adhesiveness removal treatments. The asterisks indicate significant difference ($p < 0.05$) when comparing the hatching rate to the traditional bentonite clay treatment. The letter denotes the different egg treatment method: A for bentonite clay, B for 0.075% sodium hypochlorite, C for 0.135% sodium hypochlorite, D for 0.21% sodium hypochlorite, E for 0.5% Alcalase, F for 1% Alcalase, G for 1.5% Alcalase, H for 2% Alcalase, I for 3% Alcalase, J for 5% Alcalase, K for 500 mg/L tannic acid, L for 1000 mg/L tannic acid, M for 1500 mg/L tannic acid.

4.3.3 SEM Imaging of Delta Smelt Eggs

To understand the differences between the surfaces of Delta Smelt eggs after different adhesiveness removal methods, SEM was utilized. This approach provided more details, revealing key distinctions on the surfaces of the fish eggs. Furthermore, it highlighted the potential surface structural reasons contributing to the varying adhesiveness removal across treatments. The SEM images, correlating to the conditions specified in Table 4.2, are presented in Figures 4.3 and 4.4.

At 150x magnification, surface distinctions among the eggs were subtle. However, a noticeable fungal growth in image J-1 was observed. Images from A-1 to H-1 displayed the entire egg structure, with occasional minor deformities and cracks, potentially attributed to sampling or fixation procedures.

As the magnification was increased to 2000x and 5000x, unique surface patterns began to emerge. A-2 and A-3 (simply scraped off the surface) showed a pronounced web-

like structure on the egg surface interspersed with scattered villi. For B-2, B-3, C-2, and C-3 (bentonite clay), the surfaces of the eggs were completely covered by a layer of debris, presumably from the bentonite clay, with negligible difference in the egg surface morphology between groups B and C. For D-2, D-3, E-2, and E-3 (Alcalase), the web-like structures were absent, the surface appeared smoother, and the villi were less pronounced. Some residual debris remained. For F-2, F-3, G-2, and G-3 (Tannic acid), the web-like structures disappeared, with fewer apparent villi. The surfaces of F-2 and F-3 displayed minimal debris and exhibited filamentous structures, whereas G-2 and G-3 presented an almost debris-free surface with less obvious villi compared to A-2 and A-3. For H-2, H-3, I-2, and I-3 (sodium hypochlorite), the egg surfaces lacked both debris and web-like formations. While H-3 showed less pronounced villi compared to group A to G, I-3 was entirely villi-free. Notably, pores were observed in I-3, suggesting potential surface damages. For J-2 and J-3 (dead egg with fungal growth), the images displayed obvious fungal and bacterial colonization. The egg surface showed various decomposition indicators, including surface penetration by fungi, pores, and a notably higher bacterial presence compared to all other groups.

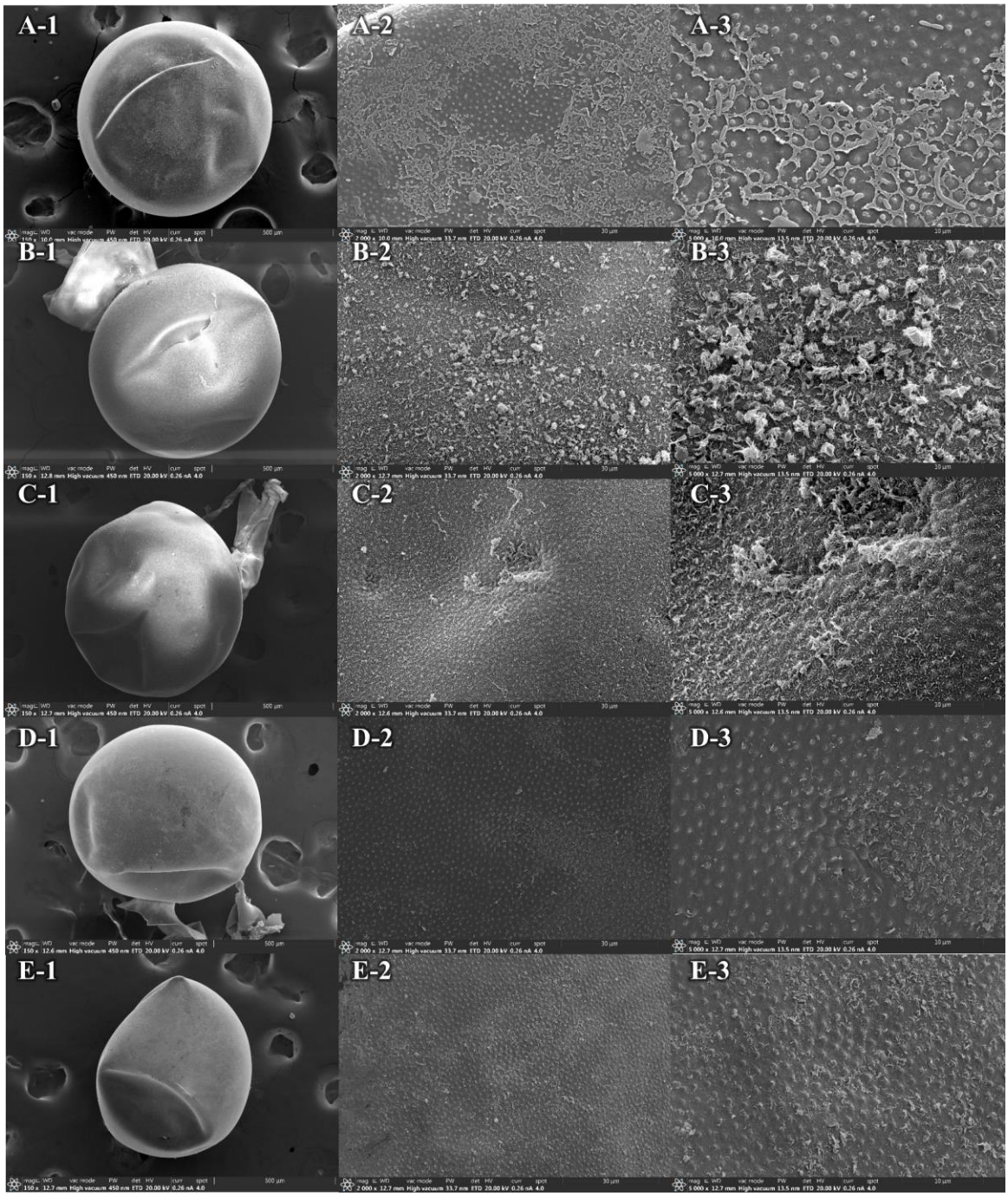

Figure 4.3 SEM images of Delta Smelt eggs (A-E). Each image is labeled with a combination of a letter followed by a number to differentiate them. The letter denotes the different egg treatment method: A for simply scraping the live egg off the surface, B for the live egg with 1 minute bentonite clay treatment, C for the dead egg with 1 minute bentonite clay treatment, D for the live egg with 2 minute 2 % Alcalase enzyme treatment, and E for the live egg with 2 minute 5 % Alcalase enzyme treatment. The number denotes the magnification level: 1 for 150x, 2 for 2000x, and 3 for 5000x.

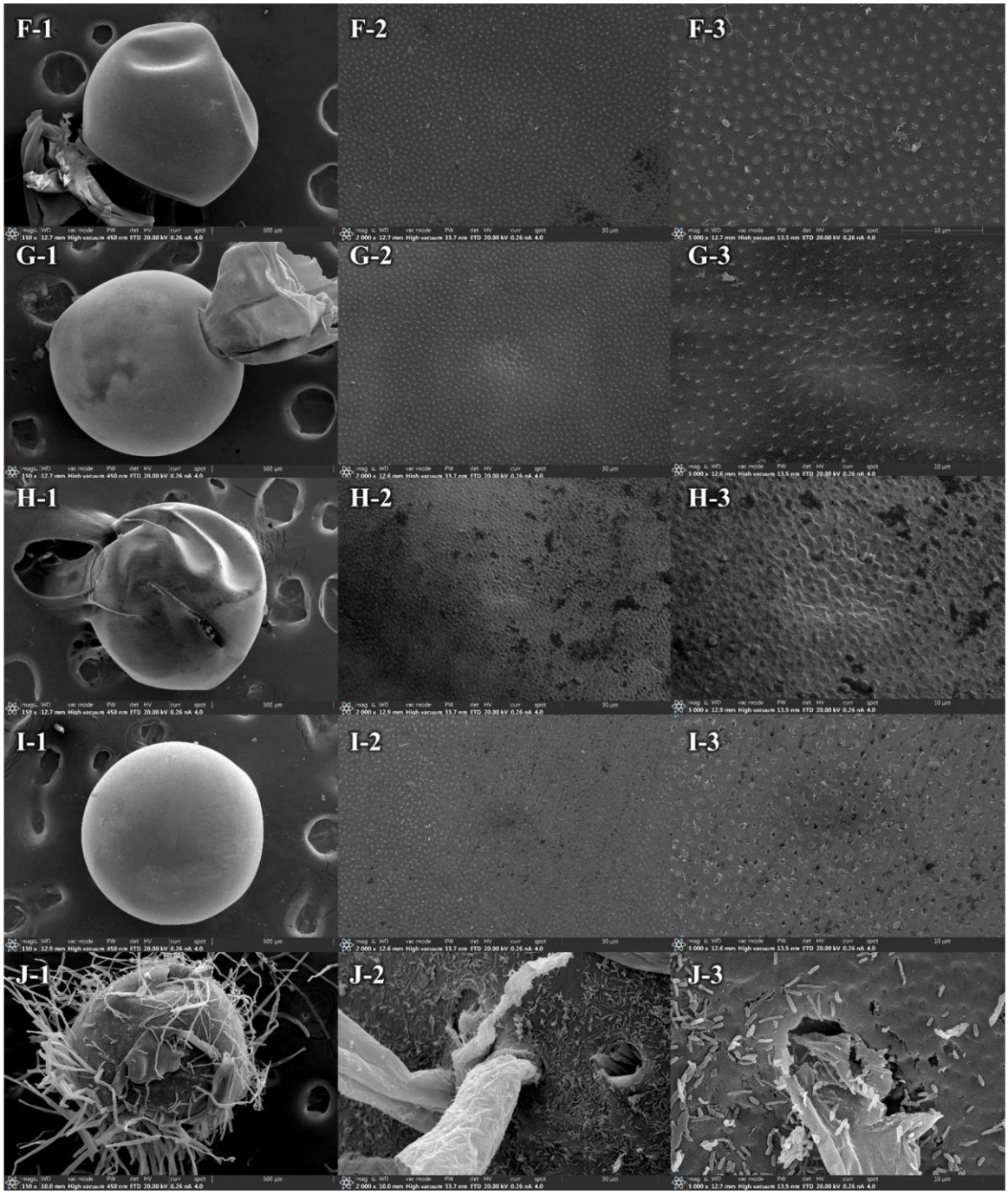

Figure 4.4 SEM images of Delta Smelt eggs (F-J). Each image is labeled with a combination of a letter followed by a number to differentiate them. The letter denotes the different egg treatment method: F for the live egg with 2 minute, 500 mg/L tannic acid treatment, G for the live egg with 2 minute 1500 mg/L tannic acid treatment, H for the live egg with 2 minute 0.075% sodium hypochlorite treatment, I for the live egg with 2 minute 0.2% sodium hypochlorite treatment, and J for simply scraping the dead egg with fungal growth off the surface. The number denotes the magnification level: 1 for 150x, 2 for 2000x, and 3 for 5000x.

4.4 Discussion

4.4.1 Sodium Hypochlorite: Effective Adhesiveness Removal but with a Cost

The application of sodium hypochlorite demonstrated an undeniable ability to remove the adhesiveness of Delta Smelt eggs. However, the hatching rates were notably poor across all concentrations of the sodium hypochlorite solution. It is worth noting that the effectiveness of sodium hypochlorite as an adhesiveness removal agent varies among species. For instance, according to the study conducted by Pšenička in 2016, Sterlet Sturgeon (*Acipenser ruthenus*) eggs had their adhesiveness removed using a mere 0.03% sodium hypochlorite solution in just 40 seconds. This method was also determined suitable for Siberian Sturgeon (*Acipenser baerii*) and Danube Sturgeon (*Acipenser gueldenstaedtii*). In the case of Wels Catfish, a similar concentration of 0.3 mg/L sodium hypochlorite solution was also effective within 40 seconds (Pšenička & Franěk, 2021). However, in another study conducted by Bian et al. in 2022, Greenfin Horse-faced Filefish (*Thamnaconus septentrionalis*) eggs remained adhesive even after a prolonged exposure to a high concentration (500 mg/L) sodium hypochlorite solution. In this same study of Bian et al., they highlighted that the efficacy of sodium hypochlorite as an adhesiveness removal agent is not only species-specific but might also be influenced by the physicochemical properties of the surrounding water.

In this study with Delta Smelt, by 10 DPF, a significant decline in hatching success was evident across all groups treated with sodium hypochlorite. All eggs either failed to hatch or became opaque and died. SEM examination of the egg surfaces revealed the presence of pores on the egg surface after being treated by 0.210% sodium hypochlorite solution for 2 minutes (Figure 4.5). It is possible that these perforations allowed the sodium hypochlorite solution to infiltrate the egg, potentially affecting embryo development. However, the exact reasons for sodium hypochlorite affecting the eggs of Delta Smelt

remains unknown. Future research is required to understand the mechanisms and potential species-specific susceptibilities of sodium hypochlorite concentrations.

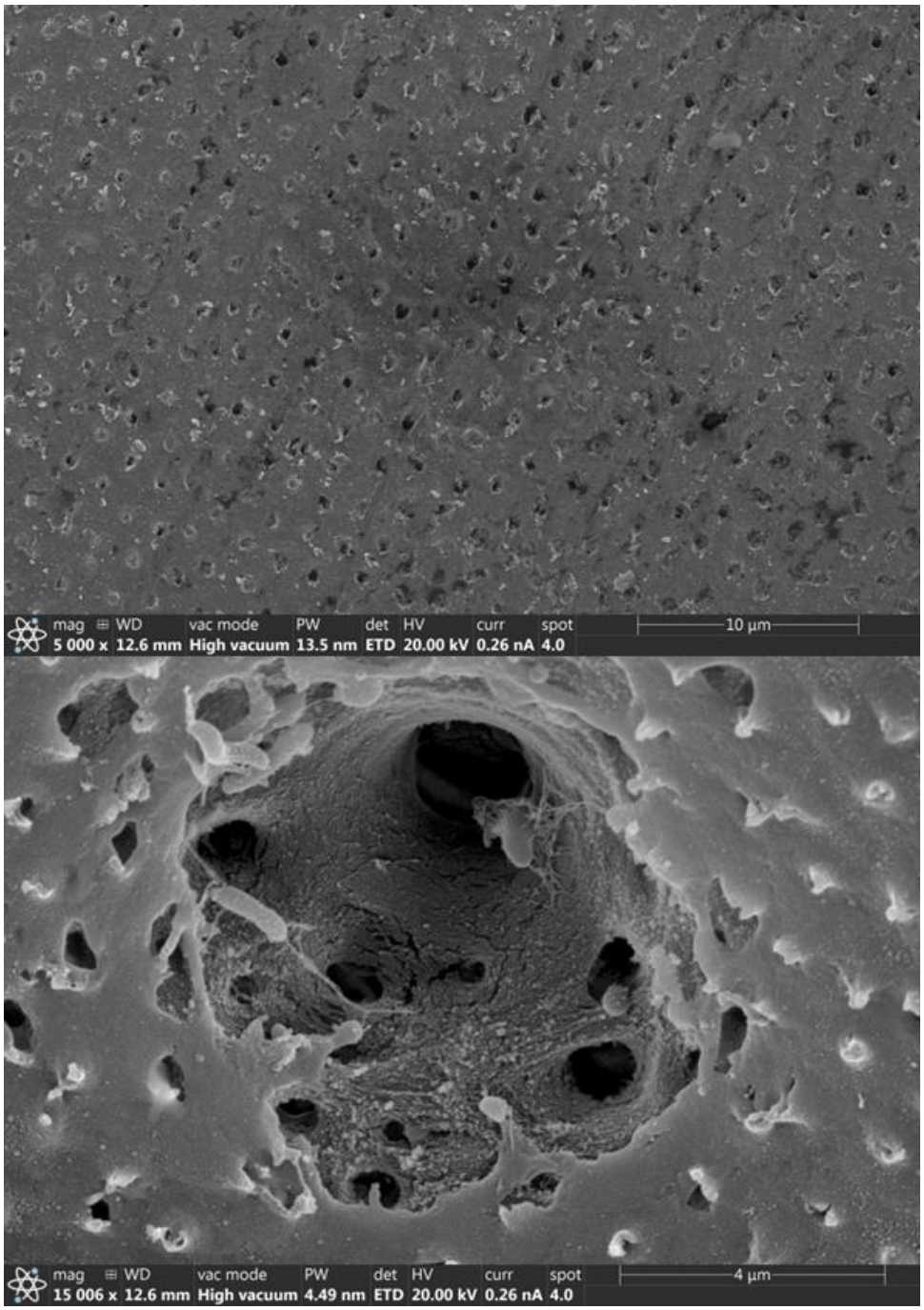

Figure 4.5 SEM images of perforations on the surface of a Delta Smelt egg treated with a 0.2% sodium hypochlorite solution. The top image shows a magnification of 5000x, while the bottom image provides a more detailed view on a different area of the same egg at 15000x magnification.

4.4.2 Proteolytic Enzyme: Balanced Adhesiveness Removal and Viability

The application of the Alcalase enzyme has shown to be a promising method for the Delta Smelt egg adhesiveness removal. In our study, while lower concentrations of the Alcalase enzyme (0.5%, 1%, 1.5%, and 2%) were found to be less effective in removing adhesiveness, higher concentrations, particularly 3% and 5%, demonstrated a marked improvement in adhesiveness removal efficacy. Notably, the 5% concentration for a 2-minute treatment emerged as the most effective in eliminating egg stickiness. Moreover, it is worth noting that the impact of the Alcalase enzyme on hatching rates is minimal, even at these higher concentrations. This suggests that while the Alcalase enzyme is effective in removing adhesiveness, it did not adversely affect the overall viability of the egg.

The variability in the efficacy of the Alcalase enzyme across different species can be attributed to the differences in egg chorion structures or the potential differences in enzyme suppliers (Bian et al., 2022). For instance, Linhart et al. (2000) demonstrated the effectiveness of the Alcalase enzyme treatment in eliminating egg stickiness in Tench (*Tinca tinca L.*) under hatchery conditions. Their results indicated a concentration dependent response in hatching rates. Specifically, a concentration of 1% Alcalase treatment resulted in the highest hatching rate of 87.1%. When the concentration was adjusted to 1.5% and 0.5%, the hatching rates hovered around 85%. However, an increase in concentration to 2.0% led to a decline in the hatching rate to 80%. In contrast, the traditional egg adhesiveness process, which employs a milk/clay treatment, resulted in a lower hatching rate of 74.1%, and it required a considerably longer treatment time of 1 hour. Comparatively, our study on Delta Smelt eggs revealed distinct findings. While Linhart et al. (2000) found optimal results at a concentration of 1%, our research indicated that lower concentrations of the Alcalase enzyme were not as effective in removing adhesiveness. It was only at higher concentrations that we observed optimal adhesiveness removal efficacy.

Remarkably, the 5% concentration for a 2-minute treatment emerged as the most effective, not only in eliminating egg stickiness but also in maintaining a same hatching rate comparable to the traditional bentonite method.

Another study by Linhart et al. (2003) also highlighted the improvement in artificial reproduction of Common Carp (*Cyprinus carpio*). Their study emphasized the optimization of various techniques, including the elimination of egg stickiness, which is the primary focus of this chapter. Their methodology involved a meticulous two-step application of enzymes, specifically α-chymotrypsin and Alcalase, to eliminate egg adhesiveness. The Alcalase treatment involved concentrations of 0.2% and 2.0% for durations ranging from 45 to 90 seconds, yielding hatching rates of 80–87%. By comparing to the traditional adhesiveness removal method of milk treatment, they found the hatching rates were similar. Comparing our findings with those of Linhart et al. (2003), it is evident that the Delta Smelt eggs responded differently to Alcalase concentrations. While Linhart et al. (2003) utilized a two-step process with the aforementioned concentrations, our study indicated that a single treatment with higher concentrations, especially 5% for 2 minutes, was effective in egg adhesiveness removal in Delta Smelt eggs. Furthermore, Linhart et al. (2003) highlighted the efficiency of enzyme treatments in shortening the traditional adhesiveness removal process in Common Carp from 70 minutes to just 21 minutes. This enzyme treatment has also been successfully applied to other species like European Catfish (*Silurus glanis* L.) and Tench, reducing egg handling time from 1 hour using traditional methods to approximately 2 minutes with enzyme treatment.

For Delta Smelt eggs, the Alcalase treatment offers multiple benefits. Not only did it maintain a commendable hatching rate, but the clarity of the enzyme solution also ensures better visibility for egg separation. Consequently, it reduced the likelihood of egg clutch formation, as any potential clusters can be identified and addressed promptly by manually

rubbing the eggs off surfaces or from each other. In contrast, with the traditional bentonite clay treatment, fish eggs are typically not visible during the process, leading to frequently observed egg clutches after adhesiveness removal. Additionally, this method reduces debris on fish egg surfaces compared to the traditional bentonite clay adhesiveness removal process. The treatment time with Alcalase was 2 minutes, which was comparable to the 1-minute duration required for the bentonite clay treatment. However, there are several considerations for using the Alcalase enzyme: Alcalase, being an enzyme, requires cooler storage conditions and has a shorter shelf life compared to the more stable bentonite clay powder that can be stored at room temperature for a long time. Future study is recommended to understand the potential impacts of Alcalase on Delta Smelt and Longfin Smelt eggs or larvae. It would also be valuable to explore if higher concentrations of Alcalase could facilitate the adhesiveness removal process while preserving the favorable hatching rates. Given that Longfin Smelt eggs are incubated at 12°C, this temperature could influence the effectiveness of the enzyme. Thus, future studies should also evaluate the impact of Alcalase enzyme treatment specifically on the eggs of Longfin Smelt and other species that are incubated in different conditions.

The critical capability of proteolytic enzymes in removing fish egg adhesiveness lies in its ability to break down the outer protein layer of the fish eggs, as highlighted by Bian et al. in 2022. The thinning of this outer layer corresponds with findings from Chapter 3, where fish eggs were discovered in the feces of Ramshorn Snails (Helisoma anceps) when they were employed as natural fish egg separators. If Alcalase can indeed degrade this outer protein layer of the eggs of Delta Smelt, future research could explore whether eggs treated with this enzyme can become more digestible for the snails and potentially with the eggs with these thinner outer layers, the eggs might release more substances that can attract the

snails. Consequently, this might offer a means to enhance the fish egg separation technique using Ramshorn Snails as natural separators.

4.4.3 Tannic Acid: Effective Adhesiveness Removal with Potential Risks

Tannic acid has been recognized for its capability to remove the adhesiveness from fish eggs, particularly in species like Pikeperch (Demska-Zakęś et al., 2005; Żarski et al., 2015). Its mechanism of action is believed to involve interactions with egg proteins, leading to co-precipitation, as highlighted in the study conducted by Kawamoto et al., in 1996. This interaction might be the reason behind its effectiveness in reducing adhesiveness. In our study, the SEM images showed the egg surface structural differences from the egg treated with bentonite clay (B-3 and C-3 in Figure 4.3, F-3 and G-3 in Figure 4.4). However, the mechanisms behind these differences for Delta Smelt are unclear. Future study might focus on the composition of the fish egg surface and mechanisms of these differences on the surfaces between different adhesiveness removal treatment methods.

In this study, tannic acid displayed consistent fish egg adhesiveness removal capabilities across all tested concentrations within the 2-minutes treatment time. The hatching rate also exhibited a promising outcome. Within the concentration and treatment durations defined in this study, tannic acid is a suitable application for Delta Smelt eggs, ensuring their viability. An additional benefit of using tannic acid for removing egg adhesiveness is its clarity. Similar to the Alcalase enzyme, tannic acid enhances visibility during the fish egg separation process. This visibility improvement increases the chances of egg clutch being seen during the process, especially when compared to the traditional bentonite clay method. These characteristics make the efficacy of tannic acid in fish egg separation similar to that of the Alcalase enzyme solution. Additionally, the storage condition for tannic acid, which is simply a dry, dark place at room temperature, makes it

a preferable option compared to the Alcalase enzyme, which requires cooler storage conditions, such as refrigeration.

However, the effectiveness of tannic acid might not be universal across all fish species. The mechanisms behind the adhesiveness of fish eggs often vary among species, and while tannic acid has shown promise in species like Pikeperch, its efficacy in other species remains to be explored further in detail. Noticeably, the study on Pikeperch also indicated that tannic acid in higher concentrations can pose a negative impact on the hatching rates (Demska-Zakęś et al., 2005). The underlying mechanisms through which tannic acid interacts with fish eggs remain a topic of interest. It is suggested that tannic acid might interact with the glycoproteins or mucopolysaccharides on the egg surface, leading to reduced adhesiveness (Hagerman et al., 1998; Kawamoto et al., 1996; Ljubobratović et al., 2017). Future study is needed to verify these interactions and their implications. In conclusion, while tannic acid presents a promising solution to the challenge of egg adhesiveness in aquaculture, its application requires careful consideration of concentration and species-specific responses to ensure optimal outcomes.

4.5 Conclusion

The quest for searching effective fish egg adhesiveness removal methods can be complicated, influenced by many factors including species-specific responses, concentration levels, and treatment durations. Our study on Delta Smelt eggs has provided valuable insights into the efficacy and potential drawbacks of three different treatment methods.

Sodium hypochlorite, although effective in reducing adhesiveness, demonstrated notable challenges to the viability of Delta Smelt eggs. While for the eggs of some species,

sodium hypochlorite can be safely applied. This highlights the varying outcomes across species, underscoring the need for more species-specific testing.

The Alcalase enzyme showed potential in balancing adhesiveness removal with minimal disruption to hatching rates. However, its effectiveness varies across species, influenced by factors like egg chorion structures. While the clear solution offers operational advantages over traditional methods like bentonite clay, it is essential to note that the enzyme may induce premature hatching, which is not always desirable. Further exploration of its applications and interactions with eggs of various species is crucial before deeming it superior to other methods.

Tannic acid, with its protein-interacting capabilities, emerged as another noteworthy option. Its clear solution aids operational visibility during the egg separation process, similar to the Alcalase enzyme. Yet, while promising, certain concentrations have shown potential adverse effects on the hatching rates of some species. It is important to conduct comprehensive testing across species to understand these effects before large-scale implementation.

In conclusion, neither the enzyme nor the tannic acid fish egg adhesiveness removal method can be definitely declared superior without more comprehensive testing. Both methods present unique advantages and require further exploration to ensure their broad applicability in aquaculture practices.

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CHAPTER 5

CONCLUSION

The study provided successful alternatives for the enhancement of fish egg separation techniques, specifically for Delta Smelt and Longfin Smelt. Two distinct methods were explored: first, utilizing Epsom salt solutions, which demonstrated the capability to separate Delta Smelt and Longfin Smelt eggs within 30 minutes without significantly affecting hatching rates, particularly in smaller-scale experiments. While large-scale applications showed decreased hatching rates for Delta Smelt, given the abundant egg production of these species and the efficiency of the method, it remains recommended for fish egg separation. The second method incorporated Ramshorn Snail as a natural separator. This method showed promise as the snails selectively consumed dead Delta Smelt eggs over live ones, though further optimization is needed for large-scale application. In order to further strengthen the new alternative fish egg separation techniques, the study investigated the removal of egg adhesiveness, by comparing sodium hypochlorite, proteolytic enzyme, and tannic acid. Sodium hypochlorite was hazardous to the eggs, preventing successful hatching. In contrast, proteolytic enzyme and tannic acid proved to be harmless, greatly enhancing the visibility during treatment and resulting in less debris than the traditional bentonite method. These two methods are recommended for future application. Further study is required to explore the broader applicability of these methods across species and to identify any potential impacts to the fish. In the end, this study aims to enhance aquaculture practices for Delta Smelt and Longfin Smelt. The aspiration is that these refined methods will contribute to the conservation of these endangered species and find utility in the broader aquaculture industry.

APPENDICES

Appendix A: Statistical Analysis R code for 2.2.5.1 Density Separation

```
# Load required libraries for statistical analyses
library(car)
library(pscl)
library(rcompanion)
library(multcompView)
library(emmeans)
library(multcomp)

# Set working directory to the specified path
setwd("C:\\Users\\ Desktop")

# Read the dataset from a CSV file into a data frame
data <- read.table("DS separation.csv", header=TRUE, sep=",")

# Display the column names of the data
names(data)

# Convert the SG_Value column to a factor variable with specific levels
data$SG_Value<- factor(as.factor(data$SG_Value), levels=c("1.04","1.06","1.08","1.1",
                                    "1.12","1.14","1.16","1.18"))

# Display the levels of the factorized SG_Value column
levels(as.factor(data$SG_Value))

# Fit a Poisson generalized linear model with Time as response and interaction of
Egg_Type and SG_Value as predictors
model.p = glm(Time ~ Egg_Type*SG_Value, family = poisson, data = data)

# Display summary statistics for the fitted model
summary(model.p)

# Perform Type II analysis of variance on the fitted model using likelihood ratio tests
Anova(model.p,
    type="II",
    test="LR")

# Calculate Nagelkerke R-squared for the fitted model
nagelkerke(model.p)

# Compute the estimated marginal means for the combinations of Egg_Type and SG_Value
from the fitted model
marginal = emmeans(model.p,
            ~ Egg_Type*SG_Value)
```

```
# Pairwise comparisons of the estimated marginal means with Sidak adjustment
P=pairs(marginal,
        adjust="sidak")
P

# Pairwise comparisons using another method (probably pairwise comparisons for
proportions)
P=pwpm(marginal,
       adjust="sidak")
P

# Perform compact letter display on the marginal means with Sidak adjustment for multiple
comparisons
Sum = cld(marginal,
          alpha=0.05,
          Letters=letters,  ### Use lower-case letters for .group
          adjust="sidak")
Sum

######################

# Now, for the Longfin Smelt dataset:

# Set working directory to the specified path
setwd("C:\\Users\\ Desktop")

# Read the LFS dataset from a CSV file into a data frame
data <- read.table("LFS separation.csv", header=TRUE, sep=",")

# Display the column names of the data
names(data)

# Convert the SG_Value column to a factor variable with specific levels
data$SG_Value<- factor(as.factor(data$SG_Value), levels=c("1.04","1.06","1.08","1.1",
                                          "1.12","1.14","1.16","1.18"))

# Display the levels of the factorized SG_Value column
levels(as.factor(data$SG_Value))

# Fit a Poisson generalized linear model with Time..second. as response and interaction of
Egg_Type and SG_Value as predictors
model.p = glm(Time..second. ~ Egg_Type*SG_Value, family = poisson, data = data)

# Display summary statistics for the fitted model
summary(model.p)

# Perform Type II analysis of variance on the fitted model using likelihood ratio tests
Anova(model.p,
      type="II",
      test="LR")
```

```
# Calculate Nagelkerke R-squared for the fitted model
nagelkerke(model.p)

# Compute the estimated marginal means for the combinations of Egg_Type and SG_Value
from the fitted model
marginal = emmeans(model.p,
                   ~ Egg_Type*SG_Value)

# Pairwise comparisons of the estimated marginal means with Sidak adjustment
P=pairs(marginal,
        adjust="sidak")
P

# Pairwise comparisons using another method (probably pairwise comparisons for
proportions)
P=pwpm(marginal,
       adjust="sidak")
P

# Perform compact letter display on the marginal means with Sidak adjustment for multiple
comparisons
Sum = cld(marginal,
          alpha=0.05,
          Letters=letters,  ### Use lower-case letters for .group
          adjust="sidak")
Sum
```

Appendix B: Box Plots R code for Figure 2.6 Settlement time for live and dead Delta Smelt eggs at various specific gravity values, and Figure 2.7 Settlement time for live and dead Longfin Smelt eggs at various specific gravity values

```
# Load necessary libraries
library(ggplot2)
library(scales)
library(ggh4x)

# Define functions to calculate standard error (SE) and the minimum and maximum SE
se <- function(x) sd(x) / sqrt(length(x))
se.min <- function(x) mean(x) - se(x)
se.max <- function(x) mean(x) + se(x)

# Delta Smelt
# Read the data from the CSV file into a dataframe
data <- read.table("DS separation.csv", header=TRUE, sep=",")
# View the names (columns) of the dataset
names(data)

# Order specific gravity value as a factor with specified levels
data$SG_Value <- factor(as.factor(data$SG_Value), levels=c("1.04","1.06", "1.08","1.1","1.12","1.14","1.16","1.18"))

# Generate boxplot of time against specific gravity value for different Egg_Types using the Delta Smelt dataset
p <-ggplot(data, aes(y=Time, x=SG_Value, color=Egg_Type, fill = Egg_Type, shape=Egg_Type)) +
  geom_boxplot(alpha=0.7,linetype=1, width=0.7, size=0.5, outlier.shape = NA)+
  xlab (expression(paste(bold("Specific gravity", sep=""))))+
  ylab (expression(paste(bold("Sinking time (sec)", sep=""))))+
  scale_y_continuous(breaks=seq(0, 1800, 200), limits=c(0, 1800))+
  theme(text=element_text(family="serif"))+ #, face="bold", size=12
  scale_fill_manual(values =c('#F8766D','#619CFF'))+
  scale_color_manual(values =c('#F8766D','#619CFF'))+
  scale_shape_manual(values =c(21,24))+
  theme(legend.title = element_text(colour="black", size=20, face="bold"))+
  theme(legend.text = element_text(colour="black", size=16))+
  theme(legend.background = element_blank())+
  theme(legend.key = element_blank())+
  theme(legend.box.background = element_blank())+
  theme(legend.key.size = unit(1.5, "lines"),legend.key.width = unit(0.5,"cm"))+
  theme(legend.position="right",legend.direction ="vertical",legend.box = "vertical")+ # add legend on top
  theme(strip.text.x = element_text(size = 16, colour = "black"))+
  theme(text = element_text(size = 20,color="black", face="bold"),
        axis.title = element_text(size = 20,color="black", face="bold"),
        axis.text = element_text(size = 16,color="black", face="bold"),
```

```
      axis.line.x = element_line(color="black", size = 0.5),
      axis.line.y = element_line(color="black", size = 0.5),
      panel.grid.major = element_blank(),# remove grid line within the plot
      panel.grid.minor = element_blank(),
      panel.border = element_blank(),
      panel.background = element_blank(),
      plot.background = element_rect(fill = "NA"))

p

# Enhance the legend and make it more descriptive
p2<-p+ guides(fill=guide_legend("Egg type"),
           color = guide_legend("Egg type"),
           size = guide_legend("Egg type"),
           shape = guide_legend("Egg type"))

p2

# Print the final plot
print(p2)

########################################

# Same plotting for the Longfin Smelt dataset
data <- read.table("LFS separation.csv", header=TRUE, sep=",")

# View the names (columns) of the dataset
names(data)

# Order SG_Value as a factor for the Longfin Smelt dataset in the same way as before
data$SG_Value <- factor(as.factor(data$SG_Value), levels=c("1.04","1.06",
"1.08","1.1","1.12","1.14","1.16","1.18"))

# Generate the boxplot for the Longfin Smelt dataset
p <-ggplot(data, aes(y=Time..second., x=SG_Value, color=Egg_Type, fill = Egg_Type,
shape=Egg_Type)) +
  geom_boxplot(alpha=0.7,linetype=1, width=0.7, size=0.5, outlier.shape = NA)+
  xlab (expression(paste(bold("Specific gravity", sep=""))))+
  ylab (expression(paste(bold("Sinking time (sec)", sep=""))))+
  scale_y_continuous(breaks=seq(0, 1800, 200), limits=c(0, 1800))+
  theme(text=element_text(family="serif"))+ #, face="bold", size=12
  scale_fill_manual(values =c('#F8766D','#619CFF'))+
  scale_color_manual(values =c('#F8766D','#619CFF'))+
  scale_shape_manual(values =c(21,24))+
  theme(legend.title = element_text(colour="black", size=20, face="bold"))+
  theme(legend.text = element_text(colour="black", size=16))+
  theme(legend.background = element_blank())+
  theme(legend.key = element_blank())+
  theme(legend.box.background = element_blank())+
  theme(legend.key.size = unit(1.5, "lines"),legend.key.width = unit(0.5,"cm"))+
```

```
  theme(legend.position="right",legend.direction ="vertical",legend.box = "vertical")+ #
add legend on top
  theme(strip.text.x = element_text(size = 16, colour = "black"))+
  theme(text = element_text(size = 20,color="black", face="bold"),
        axis.title = element_text(size = 20,color="black", face="bold"),
        axis.text = element_text(size = 16,color="black", face="bold"),
        axis.line.x = element_line(color="black", size = 0.5),
        axis.line.y = element_line(color="black", size = 0.5),
        panel.grid.major = element_blank(),# remove grid line within the plot
        panel.grid.minor = element_blank(),
        panel.border = element_blank(),
        panel.background = element_blank(),
        plot.background = element_rect(fill = "NA"))

p

# Enhance the legend for the Longfin Smelt dataset plot
p2<-p+ guides(fill=guide_legend("Egg type"),
              color = guide_legend("Egg type"),
              size = guide_legend("Egg type"),
              shape = guide_legend("Egg type"))
p2

# Print the final plot for the Longfin Smelt dataset
print(p2)
```

Appendix C: Statistical Analysis R code for 2.2.5.2.1 Small-Scale Experiments Analysis

```
# Load required libraries
library(tidyverse)
library(car)
library(rstatix)

# Set the working directory to a specified path
setwd("C:\\Users\\ Desktop")

# Read datasets from csv files into data frames
ds_data <- read.csv("DS separation hatching.csv")
lfs_data <- read.csv("LFS separation hatching.csv")

# Check the normality of the residuals for a two-way ANOVA using the Shapiro-Wilk test for the Delta Smelt dataset
ds_model <- aov(Hatching_Rate_percentage ~ SG_Value * Treatment_Time_minutes, data = ds_data)
ds_shapiro <- shapiro.test(resid(ds_model))
print(ds_shapiro)
# Check the normality of the residuals for a two-way ANOVA using the Shapiro-Wilk test for the Longfin Smelt dataset
lfs_model <- aov(Hatching_Rate_percentage ~ SG_Value * Treatment_Time_minutes, data = lfs_data)
lfs_shapiro <- shapiro.test(resid(lfs_model))
print(lfs_shapiro)

# Levene's test for checking the homogeneity of variance of the Delta Smelt dataset for each factor separately
ds_levene_sg <- leveneTest(Hatching_Rate_percentage ~ as.factor(SG_Value), data = ds_data)
ds_levene_time <- leveneTest(Hatching_Rate_percentage ~ as.factor(Treatment_Time_minutes), data = ds_data)
print(ds_levene_sg)
print(ds_levene_time)

# Levene's test for checking the homogeneity of variance of the Longfin Smelt dataset for each factor separately
lfs_levene_sg <- leveneTest(Hatching_Rate_percentage ~ as.factor(SG_Value), data = lfs_data)
lfs_levene_time <- leveneTest(Hatching_Rate_percentage ~ as.factor(Treatment_Time_minutes), data = lfs_data)
print(lfs_levene_sg)
print(lfs_levene_time)

# Kruskal-Wallis Test to test for significant differences in median hatching rates across different SG_Values for Delta Smelt dataset
ds_kruskal_sg <- kruskal.test(Hatching_Rate_percentage ~ SG_Value, data = ds_data)
```

```
print(ds_kruskal_sg)

# Kruskal-Wallis Test to test for significant differences in median hatching rates across different Treatment_Times for Delta Smelt dataset
ds_kruskal_time <- kruskal.test(Hatching_Rate_percentage ~ Treatment_Time_minutes, data = ds_data)
print(ds_kruskal_time)

# Kruskal-Wallis Test to test for significant differences in median hatching rates across different SG_Values for Longfin Smelt dataset
lfs_kruskal_sg <- kruskal.test(Hatching_Rate_percentage ~ SG_Value, data = lfs_data)
print(lfs_kruskal_sg)

# Kruskal-Wallis Test to test for significant differences in median hatching rates across different Treatment_Times for Longfin Smelt dataset
lfs_kruskal_time <- kruskal.test(Hatching_Rate_percentage ~ Treatment_Time_minutes, data = lfs_data)
print(lfs_kruskal_time)
```

Appendix D: Statistical Analysis R code for 2.2.5.2.2 Large-Scale Production Analysis

```
# The codes are the same for both Delta Smelt and Longfin Smelt
# Load required libraries for data processing, testing, and visualization
library(tcltk)
library(extrafont)
library(ggplot2)
library(readr)
library(dplyr)

# Set the working directory to a specified path
setwd("C:\\Users\\ Desktop")

# Read datasets from csv files into data frames
epsom_salt_data <- read.table("Large scale production epsom salt.csv", header=TRUE, sep=",")
hand_picked_data <- read.table("Large scale production hand picked.csv", header=TRUE, sep=",")

# Convert hatching rate columns to numeric and remove non-numeric or missing values
hatching_rate_epsom_salt <- as.numeric(epsom_salt_data$"Hatching.rate")
hatching_rate_hand_picked <- as.numeric(hand_picked_data$"Hatching.rate")
hatching_rate_epsom_salt <- hatching_rate_epsom_salt[!is.na(hatching_rate_epsom_salt)]
hatching_rate_hand_picked <- hatching_rate_hand_picked[!is.na(hatching_rate_hand_picked)]

# Conduct a Shapiro-Wilk test to assess the normality of the hatching rates for both methods
shapiro_test_epsom_salt <- shapiro.test(hatching_rate_epsom_salt)
shapiro_test_hand_picked <- shapiro.test(hatching_rate_hand_picked)
print(shapiro_test_epsom_salt)
print(shapiro_test_hand_picked)

# Since the data isn't normally distributed, use a Mann-Whitney U test to compare the two distributions
mw_test <- wilcox.test(hatching_rate_epsom_salt, hatching_rate_hand_picked)
print(mw_test)

# Create a boxplot to visually compare hatching rates between Epsom Salt and Hand Picked methods
# Consolidate data for the boxplot
box_data <- data.frame(
  Value = c(hatching_rate_epsom_salt, hatching_rate_hand_picked),
  Method = factor(rep(c("Epsom Salt", "Hand Picked"), each=length(hatching_rate_epsom_salt)))
)

# Plot the boxplot using ggplot2
ggplot(box_data, aes(x=Method, y=Value, fill=Method)) +
  geom_boxplot(alpha=0.7, linetype=1, width=0.25, size=0.5, outlier.shape = NA) +
  scale_fill_manual(values=c("Epsom Salt"="#F8766D", "Hand Picked"="#619CFF")) +
```

```
  scale_y_continuous(breaks = seq(0, 100, 10), limits=c(0, 100)) +
  labs(title="Comparison of Hatching Rates: Epsom Salt vs. Hand Picked",
       y="Hatching Rate (%)", x="Method") +
  theme_minimal() + # Apply a minimal theme to the plot
  theme(
    plot.title = element_text(hjust = 0.5, size = 20, face = "bold", family = "Times New Roman"),
    axis.line.x = element_line(color="black", size = 1),
    axis.line.y = element_line(color="black", size = 1),
    axis.title.x = element_text(size = 18, face = "bold", family = "Times New Roman"),
    axis.title.y = element_text(size = 18, face = "bold", family = "Times New Roman"),
    axis.text.x = element_text(size = 14, face = "bold", family = "Times New Roman"),
    axis.text.y = element_text(size = 14, face = "bold", family = "Times New Roman"),
    panel.grid.major = element_blank(),
    panel.grid.minor = element_blank(),
    panel.border = element_blank(),
    panel.background = element_blank(),
    plot.background = element_rect(fill = "NA"),
    legend.position = "none" # Remove legend from the plot
  )
```

Appendix E: Statistical Analysis R code for 3.2.5 Statistical analysis

```
# Load required libraries for data processing, testing, and visualization
library(tcltk)
library(extrafont)
library(ggplot2)
library(readr)
library(dplyr)

# Load the data
setwd("C:\\Users\\ Desktop")
data <- read.table("Snails data.csv", header=TRUE, sep=",")
names(data)

# 1. Testing Normality

# Calculate rate of change for live and dead eggs for each trial group
data <- data %>% group_by(`Trial.group`) %>%
  mutate(live_egg_diff = c(NA, diff(`Live.Eggs`)),
         dead_egg_diff = c(NA, diff(`Dead.Eggs`)))

# Shapiro-Wilk test for normality
shapiro_live <- shapiro.test(na.omit(data$live_egg_diff))
shapiro_dead <- shapiro.test(na.omit(data$dead_egg_diff))
print(shapiro_live)
print(shapiro_dead)

# 2. Mann-Whitney U Test (Experimental Groups)

# Filter for experimental groups
experimental_groups <- filter(data, `Snail.number` != 0)

# Mann-Whitney U test for experimental groups
test_live_experimental <- wilcox.test(experimental_groups$live_egg_diff,
experimental_groups$dead_egg_diff, paired = TRUE, exact = FALSE)
print(test_live_experimental)

# 3. Mann-Whitney U Test (All Groups)

# Mann-Whitney U test for all groups
test_live_all <- wilcox.test(data$live_egg_diff, data$dead_egg_diff, paired = TRUE, exact
= FALSE)
print(test_live_all)

# 4. Visualization

# Setting up data for rate of change in live eggs
box_data_live <- data.frame(
  Value = data$live_egg_diff,
  Method = factor(ifelse(data$`Snail.number` == 0, "Without Snail", "With Two Snails"))
```

```
)

# Boxplot for rate of change in live eggs
ggplot(box_data_live, aes(x=Method, y=Value, fill=Method)) +
  geom_boxplot(alpha=0.7, linetype=1, width=0.25, size=0.5, outlier.shape = NA) +
  scale_fill_manual(values=c("Without Snail"="#F8766D", "With Two Snails"="#619CFF")) +
  scale_y_continuous(breaks = seq(-15, 15, 3), limits=c(-15, 15))+
  labs(title="Rate of Change in Live Eggs: Without Snail vs. With Two Snails",
       y="Rate of Change (eggs/day)", x="Presence of Snails") +
  theme_minimal() +
  theme(
    plot.title = element_text(hjust = 0.5, size = 20, face = "bold", family = "Times New Roman"),
    axis.line.x = element_line(color="black", size = 1),
    axis.line.y = element_line(color="black", size = 1),
    axis.title.x = element_text(size = 18, face = "bold", family = "Times New Roman"),
    axis.title.y = element_text(size = 18, face = "bold", family = "Times New Roman"),
    axis.text.x = element_text(size = 14, face = "bold", family = "Times New Roman"),
    axis.text.y = element_text(size = 14, face = "bold", family = "Times New Roman"),
    panel.grid.major = element_blank(),
    panel.grid.minor = element_blank(),
    panel.border = element_blank(),
    panel.background = element_blank(),
    plot.background = element_rect(fill = "NA"),
    legend.position = "none"
  )

# Setting up data for rate of change in dead eggs
box_data_dead <- data.frame(
  Value = data$dead_egg_diff,
  Method = factor(ifelse(data$`Snail.number` == 0, "Without Snail", "With Two Snails"))
)

# Boxplot for rate of change in dead eggs
ggplot(box_data_dead, aes(x=Method, y=Value, fill=Method)) +
  geom_boxplot(alpha=0.7, linetype=1, width=0.25, size=0.5, outlier.shape = NA) +
  scale_fill_manual(values=c("Without Snail"="#F8766D", "With Two Snails"="#619CFF")) +
  scale_y_continuous(breaks = seq(-15, 15, 3), limits=c(-15, 15))+
  labs(title="Rate of Change in Dead Eggs: Without Snail vs. With Two Snails",
       y="Rate of Change (eggs/day)", x="Presence of Snails") +
  theme_minimal() +
  theme(
    plot.title = element_text(hjust = 0.5, size = 20, face = "bold", family = "Times New Roman"),
    axis.line.x = element_line(color="black", size = 1),
    axis.line.y = element_line(color="black", size = 1),
    axis.title.x = element_text(size = 18, face = "bold", family = "Times New Roman"),
    axis.title.y = element_text(size = 18, face = "bold", family = "Times New Roman"),
```

```
  axis.text.x = element_text(size = 14, face = "bold", family = "Times New Roman"),
  axis.text.y = element_text(size = 14, face = "bold", family = "Times New Roman"),
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  panel.border = element_blank(),
  panel.background = element_blank(),
  plot.background = element_rect(fill = "NA"),
  legend.position = "none"
)
```

Appendix F: Statistical Analysis R code for 4.2.8.1 Adhesiveness Removal Effectiveness across Treatment Methods

```
# Load required libraries for data processing, testing, and visualization
library(dunn.test)
library(dplyr)
library(ggplot2)
library(extrafont)
library(car)

# Load the data
setwd("C:\\Users\\Desktop ")
data <- read.csv("Detachment combine.csv")
names(data)

# Conduct the Kruskal-Wallis test
kruskal_test <- kruskal.test(`Adhesiveness.removal.grade` ~ Treatment, data=data)
print(kruskal_test)

# Conduct post-hoc pairwise comparisons using Dunn's test
dunn_result <- dunn.test(data$`Adhesiveness.removal.grade`, g=data$Treatment, method="bonferroni")

# Print pairwise comparisons
print(dunn_result)

# Find the indices of comparisons involving Bentonite Clay
clay_indices <- grep("Bentonite Clay", dunn_result$comparisons)

# Extract the adjusted p-values for these comparisons
clay_p_values <- dunn_result$P.adjusted[clay_indices]

# Combine the comparisons and their respective p-values
clay_comparisons <- data.frame(Comparison = dunn_result$comparisons[clay_indices], P_Value = clay_p_values)

# Filter the comparisons where p-value is less than 0.05
significant_clay_comparisons <- clay_comparisons[clay_comparisons$P_Value < 0.05, ]

print(significant_clay_comparisons)

# Calculate the median adhesiveness removal grade for each treatment
median_adhesiveness <- data %>%
  group_by(Treatment) %>%
  summarise(median_grade = median(Adhesiveness.removal.grade, na.rm=TRUE))

# Correctly reorder the levels of Treatment based on the treatments present in the dataset
levels_order <- c("Bentonite Clay",
```

```
            "Sodium hypochlorite 0.075%", "Sodium hypochlorite 0.135%", "Sodium hypochlorite 0.21%",
            "Alcalase 0.5%", "Alcalase 1%", "Alcalase 1.5%", "Alcalase 2%", "Alcalase 3%", "Alcalase 5%",
            "Tannic acid 500 mg/L", "Tannic acid 1000 mg/L", "Tannic acid 1500 mg/L")
data$Treatment <- factor(data$Treatment, levels = levels_order)
median_adhesiveness$Treatment <- factor(median_adhesiveness$Treatment, levels = levels_order)
new_labels <- setNames(LETTERS[1:length(levels_order)], levels_order)

# Update the color palette based on the reordered treatments
my_palette <- c(
  "Bentonite Clay" = "#D3D3D3",
  "Sodium hypochlorite 0.075%" = "#B0E57C", "Sodium hypochlorite 0.135%" = "#7CCB44", "Sodium hypochlorite 0.21%" = "#4AA02C",
  "Alcalase 0.5%" = "#AECBFF", "Alcalase 1%" = "#8CB2FF", "Alcalase 1.5%" = "#6A99FF",
  "Alcalase 2%" = "#487FFF", "Alcalase 3%" = "#2766FF", "Alcalase 5%" = "#054CFF",
  "Tannic acid 500 mg/L" = "#FF9E8A", "Tannic acid 1000 mg/L" = "#FF876D", "Tannic acid 1500 mg/L" = "#FF6F50"
)

# Median Bar Plot
p <- ggplot(median_adhesiveness, aes(x=Treatment, y=median_grade, fill=Treatment)) +
  geom_bar(stat="identity") +
  scale_fill_manual(values=my_palette) +
  scale_x_discrete(labels = new_labels) +
  labs(title="Average Adhesiveness Removal Grade by Treatment", x="Treatment Method", y="Adhesiveness Removal Grade") +
  theme_minimal() +
  theme(
    plot.title = element_text(hjust = 0.5, size = 20, face = "bold", family = "Times New Roman"),
    axis.line.x = element_line(color="black", size = 1),
    axis.line.y = element_line(color="black", size = 1),
    axis.title.x = element_text(size = 28, face = "bold", family = "Times New Roman"),
    axis.title.y = element_text(size = 28, face = "bold", family = "Times New Roman"),
    axis.text.x = element_text(size = 28, face = "bold", family = "Times New Roman", hjust = 1),
    axis.text.y = element_text(size = 28, face = "bold", family = "Times New Roman"),
    panel.grid.major = element_blank(),
    panel.grid.minor = element_blank(),
    panel.border = element_blank(),
    panel.background = element_blank(),
    plot.background = element_rect(fill = "NA"),
    legend.title = element_blank(),
    legend.text = element_text(size=14, face="bold", family="Times New Roman"),
    legend.position="none"
  )
print(p)
```

```
#####Box plot
# Plotting
p <- ggplot(data, aes(x=Treatment, y=`Adhesiveness.removal.grade`, fill=Treatment)) +
  geom_boxplot(lwd=1, color="black") +
  scale_fill_manual(values=my_palette) +
  scale_x_discrete(labels = new_labels) +
  labs(title="Adhesiveness Removal Grade by Treatment", x="Treatment Method",
y="Adhesiveness Removal Grade") +
  theme_minimal() +
  theme(
    plot.title = element_text(hjust = 0.5, size = 20, face = "bold", family = "Times New
Roman"),
    axis.line.x = element_line(color="black", size = 1),
    axis.line.y = element_line(color="black", size = 1),
    axis.title.x = element_text(size = 28, face = "bold", family = "Times New Roman"),
    axis.title.y = element_text(size = 28, face = "bold", family = "Times New Roman"),
    axis.text.x = element_text(size = 28, face = "bold", family = "Times New Roman", hjust
= 1),
    axis.text.y = element_text(size = 28, face = "bold", family = "Times New Roman"),
    panel.grid.major = element_blank(),
    panel.grid.minor = element_blank(),
    panel.border = element_blank(),
    panel.background = element_blank(),
    plot.background = element_rect(fill = "NA"),
    legend.title = element_blank(),
    legend.text = element_text(size=14, face="bold", family="Times New Roman"),
    legend.position="none"
  )
print(p)
```

Appendix F: Statistical Analysis R code for 4.2.8.2 Hatching Rate in Relation to Treatment Methods

```
# Load required libraries for data processing, testing, and visualization
library(dunn.test)
library(PMCMRplus)
library(car)
library(ggplot2)
library(extrafont)

# Load the data
setwd("C:\\Users\\Jimmy\\Desktop\\Thesis\\R")
data <- read.csv("Detachment combine.csv")
names(data)


# Calculate the residuals for the ANOVA
residuals <- data$`Hatching.Rate....` - ave(data$`Hatching.Rate....`, data$Treatment,
FUN=mean)

# Check for normality of residuals using the Shapiro-Wilk test
shapiro_test <- shapiro.test(residuals)
print(shapiro_test)

# Check for homogeneity of variances using Levene's test
levene_test <- leveneTest(`Hatching.Rate....` ~ Treatment, data=data)
print(levene_test)

# Conduct the Kruskal-Wallis test
kruskal_test <- kruskal.test(`Hatching.Rate....` ~ Treatment, data=data)
print(kruskal_test)

# Conduct post-hoc pairwise comparisons using Dunn's test
dunn_result <- dunn.test(data$`Hatching.Rate....`, g=data$Treatment,
method="bonferroni")

# Print pairwise comparisons
print(dunn_result)

# Find the indices of comparisons involving Bentonite Clay
clay_indices <- grep("Bentonite Clay", dunn_result$comparisons)

# Extract the adjusted p-values for these comparisons
clay_p_values <- dunn_result$P.adjusted[clay_indices]

# Combine the comparisons and their respective p-values
clay_comparisons <- data.frame(Comparison = dunn_result$comparisons[clay_indices],
P_Value = clay_p_values)
```

```
# Filter the comparisons where p-value is less than 0.05
significant_clay_comparisons <- clay_comparisons[clay_comparisons$P_Value < 0.05, ]

print(significant_clay_comparisons)



# Plot
# Correctly reorder the levels of Treatment based on the treatments present in the dataset
levels_order <- c("Bentonite Clay",
                  "Sodium hypochlorite 0.075%", "Sodium hypochlorite 0.135%", "Sodium
hypochlorite 0.21%",
                  "Alcalase 0.5%", "Alcalase 1%", "Alcalase 1.5%", "Alcalase 2%", "Alcalase
3%", "Alcalase 5%",
                  "Tannic acid 500 mg/L", "Tannic acid 1000 mg/L", "Tannic acid 1500 mg/L")
new_labels <- setNames(LETTERS[1:length(levels_order)], levels_order)

data$Treatment <- factor(data$Treatment, levels = levels_order)

# Update the color palette based on the reordered treatments
my_palette <- c(
  "Bentonite Clay" = "#D3D3D3",
  "Sodium hypochlorite 0.075%" = "#FFD700", "Sodium hypochlorite 0.135%" =
"#FFA500", "Sodium hypochlorite 0.21%" = "#FF4500",
  "Alcalase 0.5%" = "#AECBFF", "Alcalase 1%" = "#8CB2FF", "Alcalase 1.5%" =
"#6A99FF",
  "Alcalase 2%" = "#487FFF", "Alcalase 3%" = "#2766FF", "Alcalase 5%" = "#054CFF",
  "Tannic acid 500 mg/L" = "#FF9E8A", "Tannic acid 1000 mg/L" = "#FF876D", "Tannic
acid 1500 mg/L" = "#FF6F50"
)



# Plotting
p <- ggplot(data, aes(x=Treatment, y=`Hatching.Rate....`, fill=Treatment)) +
  geom_boxplot(outlier.shape = NA) +
  scale_fill_manual(values=my_palette) +
  scale_x_discrete(labels = new_labels) +
    scale_y_continuous(breaks = seq(0, 100, 20), limits=c(0, 100)) +
  labs(title="Hatching Rate Across Different Treatments", x="Treatment Method",
y="Hatching Rate (%)") +
  theme_minimal() +
  theme(
    plot.title = element_text(hjust = 0.5, size = 20, face = "bold", family = "Times New
Roman"),
    axis.line.x = element_line(color="black", size = 1),
    axis.line.y = element_line(color="black", size = 1),
    axis.title.x = element_text(size = 28, face = "bold", family = "Times New Roman"),
    axis.title.y = element_text(size = 28, face = "bold", family = "Times New Roman"),
```

```
    axis.text.x = element_text(size = 28, face = "bold", family = "Times New Roman",
hjust = 1),
    axis.text.y = element_text(size = 28, face = "bold", family = "Times New Roman"),
    panel.grid.major = element_blank(),
    panel.grid.minor = element_blank(),
    panel.border = element_blank(),
    panel.background = element_blank(),
    plot.background = element_rect(fill = "NA"),
    legend.title = element_blank(),
    legend.text = element_text(size=14, face="bold", family="Times New Roman"),
    legend.position="none"
  )
print(p)
```