Rearing temperature and parentage influence development of early post-hatch Longfin smelt (*Spirinchus thaleichthys*) in captive culture

By

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Abstract

The wild abundance of Longfin smelt (*Spirinchus thaleichthys*, LFS) in the San Francisco Estuary (SFE) has declined to less than 1% of pre-1980's numbers. As a result, they have been listed as "threatened" under the California Endangered Species Act since 2009. In an effort to avoid localized species extinction, a captive culture program was created for LFS through the Fish Conservation Culture Laboratory at the University of California, Davis. Larviculture methods for LFS are still being optimized to ensure a successful culture program. For my MS thesis, I tested the impacts of rearing temperature on early post-hatch LFS larvae from three different parental clutches to identify which rearing temperatures are best for successful development of these early life stages. I reared newly fertilized embryos and post-hatch, non-feeding yolk-sac larvae in freshwater (0 ppt) at 10, 12, or 15°C until 3 days post-hatch (dph). I measured larval morphometrics (total length, eye diameter, yolk sac area), and mortality for all groups. I was the first to evaluate the incidence and severity of jaw and spinal malformations for cultured LFS. Larvae reared at 15°C were smaller (mean ± SE; 0.52 ± 0.0040 mm), significantly younger at hatch (13.1 ± 0.1 days post-fertilization; dpf), and had slightly higher mortality and increased spinal malformations compared to other rearing temperatures. Embryos reared at 10 and 12°C were of similar size (10℃: 0.59 ± 0.0030 mm, 12℃: 0.56 ± 0.0030 mm), but 10°C larvae were significantly older at hatch (10℃: 18.4 ± 0.05 dpf, 12℃: 15.8 ± 0.1 dpf). Clutch had a much more variable impact on response metrics, markedly in size, suggesting that parentage may mediate developmental responses to unfavorable environmental conditions and change. No incidences of jaw malformation and a very small percentage of spinal malformations were observed amongst all treatment groups. Overall, the novel results described in this study suggest that parentage modifies the impacts of rearing temperature on larval development and survival.

As a result, I make recommendations to broaden the impact of future LFS and other sensitive larval fish studies on conservation larviculture techniques. While our understanding of factors impeding LFS culture is limited, this study represents a significant step towards identifying more biologically efficient larviculture and conservation management techniques for this and other critically threatened fish species.

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Introduction

Longfin smelt (*Spirinchus thaleichthys,* LFS) are small (~7-13 cm at maturity) anadromous forage fish natively distributed along the Pacific coast of the United States, from California to Alaska (Sağlam et al., 2021). Historically, LFS were one of the most abundant fish species in California (Moyle 2002; Sommer et al., 2007), serving as a key energetic bridge between communities of primary producers and secondary consumers. As a result, LFS have acted as an important indicator species for overall ecosystem health and function in diverse habitats like the San Francisco Estuary (SFE) (Nobriga and Rosenfield, 2016; Lewis et al., 2020). However, over the past few decades, the previously self-sustaining SFE LFS population has been reduced to less than 1% of pre-1980 observed populations, despite no dedicated fisheries for the species. This has led to LFS being listed as "threatened" under the California Endangered Species Act since 2009 (Nobriga and Rosenfield, 2016; Hobbs et al., 2017). The decline has been largely attributed to changing environmental conditions, water flow, and predator-prey dynamics (Hobbs et al., 2017). In 2015, the California Department of Fish and Wildlife reported the first zero abundance observations for LFS in the SFE's Summer Townet Survey (Hobbs et al., 2017), necessitating urgent action to understand population decline and attempt to save the species from extinction.

In response to this dramatic decline, the University of California Davis' Fish Conservation and Culture Laboratory (FCCL), in partnership with UC Davis research groups, US Department of Fish and Wildlife, California Department of Fish and Wildlife, California Department of Water Resources, and others, have been working to establish a captive culture program for LFS species conservation. Despite these efforts, we lack a complete understanding of LFS captive culture due to the fact that methodologies are mainly modeled after successful

Delta smelt (*Hypomesus transpacificus*) methods. While Delta smelt are a genetically similar species occupying similar conditions in the SFE, they have different developmental timelines and physiological tolerance to environmental change, such as temperature (Swanson et al., 2000; Rosenfield and Baxter, 2007; Nobriga and Rosenfield, 2016; Halverson, 2022). Physiological differences between these two species are particularly evident for embryos and larvae. LFS spawn earlier in the year (primarily December - February) when water temperatures typically measure between 7 and 14.5℃ (Moyle, 2002; Nobriga and Rosenfield, 2016; Yanagitsuru et al., 2021), with field data suggesting larvae are most abundant in the range of 8-12℃ (Grimaldo et al., 2017). In contrast, Delta smelt spawn later in the year (primarily January - May) when water temperatures are between 9.2 and 17.9℃ (Baskerville-Bridges et al., 2005), with field data showing highest larval abundance between 14 and 18℃ (Bennett, 2005). Comparative laboratory studies between the two species have also illustrated lower thermal tolerance for LFS (Jeffries et al., 2016), suggesting that colder rearing temperatures are historically more optimal for LFS survival (Yanagitsuru et al., 2021). While upper thermal tolerance is one of many physiological differences between the two species, Delta smelt captive culture approaches continue to be used to inform LFS culture methodologies. Currently, mortality in early larviculture remains a bottleneck for conservation culture of LFS, necessitating more research to identify factors that may be contributing to this high larval mortality.

Similar to many other aquatic animals, the larval stage represents a period where fish are generally the most sensitive to environmental change (Fujimura and Okada, 2007; Aral et al., 2011; Honji et al., 2012; Lee et al., 2014; Zhu et al., 2018). In nature, larval fish mortality is estimated at ~99% (Hjort, 1914; Houde and Hoyt, 1987), and, even in modern aquaculture, many species experience upwards of 70% mortality within the first few days after yolk reabsorption

(Shields, 2002), at which point exogenous feeding would begin (around 5-7 days post-hatch; dph for LFS). In addition to high mortality, cultured fish tend to face well-documented issues with morphological deformities, particularly during larviculture (Cobcroft et al., 2004; Fraser and de Nys, 2005; Fitzsimmons and Perutz, 2005; Kestemont et al., 2007; Nagano et al., 2007; Boglione et al., 2009; Cobcroft and Battaglene, 2009; Dionísio et al., 2012; Berillis, 2015; Vignet et al., 2019; Kourkouta et al., 2021; Martini et al., 2021). Skeletal deformities of the jaw and spine are most common, particularly scoliosis, kyphosis, and lordosis. The exact causes of malformations are not well understood, though nutrition, heavy metal pollution, water temperature, water current velocity, genetics, bacteria, and parasites are all considered to have possible roles in aquaculture settings (Berillis, 2015). Malformed fish face lower viability in both production and conservation culture. These malformations can lead to increased mortality, reduced biological performance, decreased mobility, or production of an "unremarkable product" for consumption or unsuitable fish for supplementation (Fraser and de Nys, 2005; Berillis, 2015). As they seem to bottleneck production for many species of cultured fish, it is important to investigate the potential causes and impacts of larval malformations on overall viability of cultured LFS to inform more efficient methodologies.

The larval stage for LFS lasts only a few months - typically in the winter through early- to mid-spring. As anadromous fish, the life history of LFS changes dramatically throughout different life stages. For SFE LFS, specifically, eggs are deposited dermersally in upper estuarine areas, where water tends to be more cold and fresh. As larvae, LFS remain in the protection of estuaries before migrating to more coastal regions as juveniles and sub-adults from the summer through early winter of the first year. Coastal ocean migration to full salinity occurs for adults, some of which will return upstream the following winter for spawning (Hobbs et al., 2006; Merz

et al., 2013; Nobriga and Rosenfield, 2016). Considering the significant life history changes anadromous fish like SFE LFS undergo in such a short period of time, it is vital to investigate how early life stages interact with their environment. Such information could be used to inform captive conservation culture as well as resource management (water use, fisheries management, etc.) in historic wild habitats to best protect this species and others, especially the most vulnerable life stages.

Fish, like other ectotherms, operate best in a certain range of environmental conditions, referred to as optima (Mark et a., 2005; Pörtner and Farrell, 2008; Pörtner et al., 2010; Williams et al., 2016). Optima are the conditions in which physiological performance is maximized (Crawshaw, 1977). Understanding how optima change through life history can improve organism quality and survival outcomes in both captive culture and wild habitats. Improved growth, swimming capacity, cardiac function, increased energetic reserves, and reduced stress response are just a few examples of how operating in physiological optima can benefit developing fish (Mark et al., 2005; Sokolova et al., 2012; Jeffries et al., 2016; Williams et al., 2016; Dahlke et al., 2020; Yanagitsuru et al., 2021). Particularly in terms of temperature, fish tend to require larger proportions of their energetic budget to cope with stress involved with living outside of their optimum, leaving less energy for other processes like growth, reproduction, and maintenance of immunity (Mark et al., 2005; Sokolova et al., 2012; Jeffries et al., 2016; Williams et al., 2016; Del Rio et al., 2019; Dahlke et al., 2020). In comparison, fish grown within their ranges of thermal optima have the potential to be more energetically efficient and have higher chances of survival compared to fish grown outside of their optima. In the wild, this means larger fish, likely equipped with increased energetic resources for competition and survival. Studies have shown that rearing fish above optimum but below lethal temperatures can

be beneficial by increasing upper thermal tolerance through acclimation (Myrick and Cech Jr, 2005; Schaefer and Ryan, 2006; He et al., 2014; Moyano et al., 2017). Therefore, while fish reared above optimum temperatures may face lethal energetic trade-offs in terms of embryonic resource use and growth in the wild, aquaculture methods can help safely usher sensitive larvae through critical periods without significant mortality. The result is often fish with faster growth rates and increased tolerance to elevated temperatures (Myrick and Cech Jr, 2005; Schaefer and Ryan, 2006; He et al., 2014; Moyano et al., 2017), which could be vital in contexts of conservation aquaculture like with LFS and Delta smelt.

When faced with managing a species at immediate risk of extinction, scientists are often given minimal time and few options for restoration (Jackson, 2008). As a result, an additional challenge that fish conservation scientists face is the maintenance of genetic diversity in captive culture (Lindberg et al., 2013). For Delta smelt, this problem was addressed by the creation of a "refuge population," in which wild-origin broodstock fish were held and bred in a captive refuge setting to preserve the evolutionary potential of the population for multiple generations outside of its natural habitat (Lindberg et al., 2013). To ensure successful maintenance and tracking of genetic diversity in the refuge population, it is important that the parentage of embryos are tracked, and any possible impacts of said parentage on performance are recorded. A similar refuge population has been initiated for LFS conservation at FCCL, and, therefore, it was important for this study to analyze any possible effects of parentage (ie. difference in clutch) on early development.

The main objectives of this study were to understand the impact of rearing temperature and clutch on larval morphology, mortality, and incidence of skeletal malformation in early post-hatch LFS (0-3 days post-hatch, dph). I predicted that higher rearing temperatures would

result in smaller larvae with lower overall survival and higher incidence of skeletal deformity as more energy would be allocated to stress response mechanisms and away from growth and development. I also predicted that the impacts of rearing temperature on larval morphology, malformations, and mortality would be modified by clutch, as parentage has been shown to impact sensitivity to environmental change. Finally, I predicted that the combined effects of clutch and rearing temperature would lead to the greatest difference in measured response variables between groups. By determining the impacts of both rearing temperature and clutch on early development of LFS, I aim to better inform LFS-specific captive culture methodologies for species conservation and recovery in the face of anthropogenic climate change and natural resource exploitation.

Materials and methods

Spawning, transport, and acclimation

Longfin smelt (LFS) embryos were spawned in 1-1 or 1-2 female-male breeding pairs (hereafter referred to as clutches, Appendix A) from fish held in freshwater (FW, 0 PSU) at 12℃ at the Fish Conservation and Culture Laboratory (FCCL) in Discovery Bay, CA, USA according to methods adapted from Yanagitsuru et al. (2021). Clutches of fertilized embryos were then immediately transported back to the University of California, Davis main campus in Davis, CA, USA for acclimation and experimentation. For transit, embryos were separated by clutch in 500 mL round glass dishes with plastic lids filled with 470 mL of water and placed in styrofoam coolers with ice packs to maintain water temperature at around 12℃. Upon arrival at the Academic Surge Laboratory in Davis, all dishes, separated by clutch, went directly into a 12℃ water bath with minimal disturbance until fertilization success was confirmed at 4 days

post-fertilization (dpf). From 0-4 dpf, embryos were acclimated at 12℃ and treated daily with an anti-fungal solution (diluted Pond Rid-Ich Plus, Kordon, LLC, Haywood, CA, USA), before a water change.

At 4 dpf, each clutch was sorted and assessed for fertilization success using a light box (Gagne Porta-Trace, New York, NY, USA) following a modified protocol from Baskerville-Bridges et al. (2005), after which unfertilized eggs were removed and successfully fertilized embryos from each clutch were separated into treatment replicates (each in one, 470 mL glass dish). Replicates were of a set density (~0.2 larvae mL^{-1}, ~95-100 larvae per 470 mL replicate; adapted from Yanagitsuru et al., 2021) and placed into 220-L water baths filled to ~30% capacity and correlating to one of three different temperature treatments (10, 12, or 15℃). The number of replicates (clutch:temperature combinations) per clutch was dependent on the spawn size and fertilization success of the clutch (Appendix A), with all clutches resulting in two (clutch 1) or three replicates (clutches 2 and 3) per temperature treatment. All replicates were monitored until hatch, with water physicochemical parameters, including temperature, dissolved oxygen (YSI 556, YSI, Inc., Yellow Springs, OH, USA), salinity (EXTECH RF12 Portable Brix Refractometer, Nashua, NH, USA), pH (freshwater pH test kit, Mars Fishcare North America, Inc., Chalfont, PA, USA), and ammonia (NH_3/NH_4^+ freshwater test kit, Mars Fishcare North America, Inc., Chalfont, PA, USA) measured twice daily until the completion of the project. From 4 dpf - 3 dph, 70-90% daily water changes were made for each replicate with treatment-acclimated water, and dead larvae were removed twice daily to maintain water quality. LFS embryo production was approved by UC Davis IACUC protocol #21353 and work with early larval stages was approved by IACUC protocol #22079.

Water physicochemistry

Water physicochemistry parameters were taken for each clutch:temperature replicate (8 per temperature) at every sampling time (24 observations every 12 hours). Temperatures for each treatment group (mean ± standard error; SE) remained consistent throughout the duration of the experiment (10℃: 10.1 ± 0.06℃, 12℃: 11.9 ± 0.03℃, 15℃: 14.9 ± 0.06℃). Similarly, dissolved oxygen (DO_2, mg O_2 L^{-1}) remained consistent throughout the experiment for each treatment group (10℃: 10.8 ± 0.07 mg O_2 L^{-1}, 12℃: 9.9 ± 0.06 mg O_2 L^{-1}, 15℃: 8.5 ± 0.05 mg O_2 L^{-1}). Salinity was measured at 0 PSU for each group throughout the experiment with no measurable variation. pH for all groups ranged from 7.4-7.6. Ammonia spanned a harmless range of 0-0.25 mg L^{-1} throughout the project (El-Greisy et al., 2016). Neither clutch or replicate had any significant impact on observed water physicochemistry parameters.

Larval imaging

After at least six individuals in each replicate successfully hatched, larvae were sampled for imaging. During sampling (every 12 hours), 6-10 individual larvae were randomly selected until larvae reached 3 dph (LFS clutch 1 n = 277, LFS clutch 2 n = 465, LFS clutch 3 n = 457). Larvae were euthanized in a two-step process, with the first step being a short ice bath, followed by an overdose with MS-222 (tricaine methanesulfonate) solution (0.5 g L^{-1} buffered to pH 7.4 with $NaHCO_3$). Euthanized larvae were quickly imaged on a 0.01mm calibration slide using a Canon EO6 Rebel T6 SLF camera (Canon, Tokyo, Japan) mounted on a Leica S8APO stereomicroscope (Leica Microsystems, Chicago, IL, USA).

Morphometric analysis of images

Larval images were organized by sampling date/time, replicate, individual fish, and photo number and electronically stored for later morphometric analysis using Fiji ImageJ software (Schindelin et al., 2012; Schneider et al., 2012). ImageJ software was used to take standardized, scaled measurements of total length (TL, mm), eye diameter (ED, mm), and yolk sac area (YS, mm^2). Only raw images were used to describe the presence, type (Fitzsimmons and Perutz, 2005; Nagano et al., 2007), and severity of jaw and/or spinal deformities according to a modified scale of *0*-*iii* from Cobcroft et al. (2004). In this scale, *0* was assigned to larvae with seemingly normal development, *i* was assigned to larvae with minor deformity where some elements were abnormal but overall function was not impeded, *ii* assigned to larvae with intermediate deformity where some elements were abnormal and function may have been limited, and *iii* to larvae with severe deformity where elements were abnormal and function was severely limited or eliminated, but not fatal (Fig 1). All image analyses were performed blinded.

Statistical analyses

All statistical analyses were performed using R 1.4.1103 (R Core Team, 2009-2021). Data were tested for normality and homogeneity using the *shapiro.test* function from the *stats* package and the *leveneTest* function from the *car* package, respectively. All response data proved to be non-normal and/or heterogeneous, so nonparametric tests were utilized for all statistical analyses. Linear mixed models (LMMs) were fit to data using the maximum likelihood method with the *lme4* package (Bates et al., 2014). Tukey's multiple comparison post-hoc tests were performed with the *emmeans* package (Lenth, 2016). Percentage survival, hatch total length, mean total length, eye diameter, and yolk sac area were each fit as a response variable with

temperature, clutch, interaction of temperature and clutch, and age (dph) as fixed effects and replicate as a random effect. Hatch and fertilization success percentages were analyzed using Kruskal Wallis tests (*kruskal.test*) from the *car* package (Dalgaard, 2002). Kendall's rank correlation tau tests *(function cor.test(method = c("kendall"))*) were used with the *stats* package to determine morphological relationships between dependent variables. All figures were created using the *ggplot2* package.

Results

Fertilization success

Overall, fertilization success was variable between Longfin smelt (LFS) clutches at 14.9%, 13.8%, and 27.6%, respectively (Appendix A). Hatch success for all three clutches at all three temperature treatments ranged from 98-99%. No significant differences were seen in fertilization or hatch success between temperature or clutch groups when compared using Kruskal-Wallis tests ($p = 0.3679$).

Morphometrics

Temperature and clutch significantly affected total length (mm), eye diameter (mm), and yolk sac area (mm^2) (Table 1). Most notably, the 10℃ larvae were significantly larger than 15℃ larvae across all clutches (Fig 2). The impacts of rearing temperature on size, particularly at 15℃, were significantly different by clutch (Table 1, Fig 2), suggesting that clutch did modify the effects of rearing temperature on overall morphology.

Kendall's rank correlation tau tests showed some significant correlations between total length and yolk sac area, total length and eye diameter, and total length and age at hatch (dpf) for

all treatment groups (Table 2). Specifically, total length showed a slight negative correlation with yolk sac area and a slight positive correlation with eye diameter across all temperature treatments. Total length also showed a slight positive correlation with age at hatch for the 12 and 15℃ groups, but a slight negative correlation for the 10℃ group.

Size (total length) at hatch (0 dph) was significantly affected by temperature, as well as by the interaction between temperature and clutch, but was not significantly affected by clutch alone (Table 1, Appendix B). Average size (mm) at hatch was largest for 10℃ larvae and decreased with increasing rearing temperature (10℃: 0.59 ± 0.0030, 12℃: 0.56 ± 0.0030, 15℃: 0.52 ± 0.0040). Average size at the conclusion of the experiment (3 dph) for each temperature group was 10℃: 0.62 ± 0.0030, 12℃: 0.61 ± 0.0040, 15℃: 0.58 ± 0.0020. Age (dpf) at hatch was significantly affected by temperature, clutch, and clutch:temperature interactions (Table 1). Average age at hatch was significantly higher for 10℃ larvae than for both 12 and 15℃ larvae, and decreased with increasing rearing temperature ($p = < 0.0001$; 10℃: 18.4 ± 0.05, 12℃: 15.8 ± 0.1, 15℃: 13.1 ± 0.1). Total time encompassed from 0 dpf - 3 dph ranged from 15-22 days depending on the treatment group.

Mortality

Minimal post-hatch mortality was seen in this experiment, and no significant differences were detected in mortality between temperature or clutch treatments (Table 1). However, there were differences in mortality between interacting temperature and clutch treatments, suggesting that the impacts of rearing temperature on mortality were modified by clutch groups. Percent mortality by temperature treatment was 10℃: 11.9%, 12℃: 6.5%, and 15℃: 26.6%. Percent mortality by clutch was clutch 1: 16.6%, clutch 2: 25.4%, and clutch 3: 5.5%.

Deformities

No incidences of jaw malformation were observed in 0-3 dph LFS larvae. Spinal deformities, however, were seen in a very small percentage of treatment groups. The majority of observed spinal deformities were forms of kyphosis (Fig 1), or dorso-ventral deviation of vertebrae (Fitzsimmons and Perutz, 2005; Nagano et al., 2007). It should be noted that all sampled fish, including those sampled with obvious malformation, were only sampled if there were clear signs of sustained life (active movement, respiration, stable heartbeat, etc.) prior to euthanization. While the sample size of malformed larvae in this project did not meet the criteria to perform appropriate, nonparametric statistical significance tests, presence and severity of spinal deformities did vary somewhat between treatments (Fig 3) and clutches. Incidence of spinal deformities (*i-iii* in severity) ranged (number of individuals) within temperature groups as 10℃: 0-3, 12℃: 0-6, 15℃: 0-14, and within clutches as clutch 1: 0-2, clutch 2: 0-14, clutch 3: 0-10.

Discussion

The purpose of this study was to gain insight into the effects of rearing temperature and clutch on larval morphology, mortality, and incidence of skeletal malformation in early post-hatch (0-3 dph) Longfin smelt (LFS). I found that rearing at 15℃ produced early larvae that were significantly smaller, younger (dpf) at hatch, showed increased consumption of embryonic yolk, and had slightly higher mortality than larvae reared at 10 or 12℃. I also saw that the impacts of rearing temperature on overall size, mortality, and incidence of spinal malformations were modified by parentage. These results suggest that the interaction of rearing temperature and parentage had the largest impact on larval fish morphology, mortality, and incidence of spinal

malformations for early larval LFS.

The data presented in this study suggest that 15℃ is likely an above-optimal rearing temperature for this sensitive forage fish species during early larval development. Higher rearing temperatures had significant impacts on morphological development and resource use, and may also impact mortality and malformations immediately following hatch. Larval LFS in this study reared at 15℃ were significantly smaller (Table 1, Fig 2) and younger than fish reared at 10 or 12℃. The 15℃ reared larvae also showed significantly faster consumption of yolk sac resources (Table 2), as well as higher trending (but not significant) mortality, and slightly higher trending incidence of spinal malformations (Fig 1, Fig 3). The results are consistent with other studies performed on larval fish that show rearing at temperatures above optimum can lead to smaller overall size (Del Rio et al., 2019; Yanagitsuru et al., 2021), faster consumption of cellular resources (Crawshaw, 1977; Mark et al., 2005; Jeffries et al., 2016; Del Rio et al., 2019; Dahlke et al., 2020; Yanagitsuru et al., 2021), increased frequency of skeletal malformations (Dionísio et al., 2012; Berillis et al., 2015; Kourkouta et al., 2021), and increased mortality (Swanson et al., 2000; Williams et al., 2016; Han et al., 2020; Yanagitsuru et al., 2021). In comparison, both 10 and 12℃ were suitable temperatures for growth and development of early LFS larvae.

Since differences in mortality and skeletal malformation between rearing temperatures were non-significant, they may not later manifest in any substantial developmental divergence between groups. Laboratory studies even suggest that successful acclimation of early larvae to warmer temperatures can increase upper thermal tolerance of juveniles and adults (Myrick and Cech Jr, 2005; Schaefer and Ryan, 2006; He et al., 2014; Moyano et al., 2017). Therefore, early development at higher temperatures could provide long-term benefits to LFS, although additional information about their physiology is required before attempting this. Based on these results, I

recommend that more research be conducted on the thermal tolerance and developmental plasticity of LFS over longer periods of growth. An increased availability of data on LFS thermal tolerance through development will better allow us to adapt conservation culture methodologies to changing environmental conditions in an effort to increase population resilience. Additionally, understanding the impact of above-optimal temperature acclimation on threatened cold-water species resilience in dynamic environments like the San Francisco Estuary (SFE) can help limit resource use in situations of conservation aquaculture by minimizing the need for rapidly cooled culture water.

This study was the first to document incidence and severity of skeletal malformations in LFS. I chose to investigate rearing temperature and clutch as possible causes of skeletal malformations of early post-hatch larvae, as many conditions, including elevated water temperatures and genetics, are thought to play roles in abnormal skeletal development (Berillis, 2015). Spinal malformations were present in a small percentage of larvae, with slight variations being most pronounced in interacting clutch:temperature groups (Fig 3), suggesting rearing temperature and genetics may impact incidence of spinal deformities. As LFS larvae are most commonly seen from 8-12℃, these trends in spinal malformation align with other larval fish studies that show rearing at the upper end of a fish's thermal tolerance range can promote the occurrence of spinal abnormalities (Fitzsimmons and Perutz, 2006; Yang et al., 2016; Han et al., 2020). Studies also support the finding that parentage plays a significant role in modifying the impacts of rearing temperature on the presence of spinal malformations (Fitzsimmons and Pertuz, 2006; Evans and Neff, 2009), as similar to the trend seen here. The complete absence of jaw malformations in the current study suggests that the rearing temperatures used were not a driving cause of jaw deformation through the first few days post-hatch. Although, I highlight the

limitation of my small sample size of clutches in this study, and recommend that any future studies aim to represent as many clutches as possible to better understand the impact of parentage on malformations, especially in refuge populations. These findings align with previous research which indicates jaw deformities may be induced by other factors such as broodstock nutrition (Cobcroft et al., 2004), tank color (Cobcroft and Battaglene, 2009; Cobcroft et al., 2012), or length of time in culture (Cobcroft et al., 2001; Ma et al., 2014). Since continued rearing, and inevitable removal or death, of malformed fish provides significant financial and biological burden to aquaculture systems, it is important to identify causes of deformity in the demanding larval stages, when culture efficiency can be maximized by removal of low-developmental-quality larvae (Nagano et al., 2007). For the refuge population of SFE LFS, I specifically highlight the need for research on the causes of skeletal malformations using more clutches to help clearly illustrate the interactive effects of clutch on developmental abnormalities.

Parental (ie. broodstock) life history and genetics appear to be a more driving cause of developmental divergence, especially in refuge populations like SFE LFS. The results of this study indicate that the combined effects of rearing temperature and clutch had the largest measurable impacts on LFS larval size (Table 1, Fig 2, Appendix B), mortality, and variation in the presence of spinal malformations (Fig 3). This is consistent with larval fish studies that found interaction effects between genetics and rearing temperature are common but highly variable by environment and species (Barlow, 1961; Todd et al., 1981; Marcil et al., 2006). Other work on the genetic influences of reared larval fish has found that environmental conditions, stressor levels, and diet experienced by broodstock can significantly impact larval success (Izquierdo et al., 2001; Migaud et al., 2013; Valdebenito et al., 2013; Bobe, 2015). Since refuge populations rely on wild-caught broodstock for embryos, it is vital that I aim to understand what kind of

impact life history of broodstock can have on larval quality. For example, Panagiotaki and Geffen (1992) found that lack of genetic diversity within cultured herring populations can have significant influence on the size and developmental speed of larvae, similar to trends seen here (Fig 2, Appendix B). Additionally, studies in fish genetics indicate that both additive and non-additive genetics are important factors contributing to the fitness of captively cultured fish (Neff et al., 2011), meaning artificial breeding programs like that for LFS may not adequately preserve the genetic integrity of the population. These findings support my recommendations for more work to understand LFS broodstock health and reproductive status, as well as for further investigation of the impacts of parentage on larval development success. Advancing our understanding in these areas will not only influence more efficient LFS conservation culture, but will also provide insight for conservation management of similarly threatened fish species facing negative impacts of climate change and anthropogenic interference.

This study provides data to further understand some underlying bottlenecks that likely hinder conservation larviculture of the threatened Longfin smelt. Current SFE LFS research is limited to early life stages due to challenges with closing the captive lifecycle, although even our early life stage knowledge is narrow. Larval work has started with a focus on the impacts of salinity (Yanagitsuru et al., 2022), and temperature (Yanagitsuru et al., 2021), as well as the typical conditions that spawning broodstock and early life stages best endure in the wild (Barros et al., 2022). Here, I describe the impacts of rearing temperature and clutch on early post-hatch larvae to build support for optimal rearing conditions for this species, while also being the first to evaluate skeletal malformations in cultured LFS. The main priority for the conservation of this species is closing their lifecycle, and so I outline the need for further research to contribute to a growing knowledge of barriers impeding culture success. Since rearing temperature appears to

be a driving force of physiological performance, I first recommend further analysis of longer-term effects of thermal acclimation on LFS. Similarly, the impacts of genetic variation and parentage on fish development, malformations, and survival for captive conservation culture should be further explored. Finally, I suggest additional research be performed to understand and evaluate the reproductive quality of LFS broodstock throughout the spawning period to limit resource use and improve spawn predictability. While wide knowledge gaps still exist in LFS development and reproduction, this study represents a significant step towards a better understanding of conservation larviculture and management of this and other critically threatened fish species.

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Tables and Figures

Tables

Table 1. Linear Mixed Model statistical analyses for overall total length (mm), eye diameter (mm), yolk sac area (mm^2), size and age at hatch, and mortality. Italicized p-values represent those showing statistically significant differences (alpha = 0.05). Degrees of freedom (df) were calculated using the Satterthwaite method.

Response variable	**P-values**		
	Clutch main effect	**Temperature main effect**	**Clutch:Temperature interaction effect**
Total length (mm)	*0.0046*	*< 2.20e-16*	*1.05e-08*
Eye diameter (mm)	*0.00032*	*1.01e-08*	*0.00019*
Yolk sac area (mm^2)	*2.58e-06*	*4.93e-08*	*0.00032*
Size (total length, mm) at hatch (0 dph)	0.078	*< 2.20e-16*	*8.97e-07*
Age (dpf) at hatch (0 dph)	*6.78e-11*	*< 2.20e-16*	*4.33e-09*
Mortality	0.30	0.64	0.26

Table 2. Kendall's rank correlation test results by dependent variable interaction and temperature treatment. Separating treatments by clutch did not provide any statistically significant insight into morphological correlations. Italicized p values represent significant correlations at a given temperature. Tau values are interpreted as: 0 = no correlation, 1 = perfect positive correlation, -1 = perfect negative correlation.

Response variable 1	**Response variable 2**	**Temperature treatment (℃)**	**p value**	**tau (r_τ)**
Total length (mm)	Yolk sac area (mm^2)	10	*2.228e-07*	-0.1891
		12	*0.019*	-0.0866
		15	*1.481e-10*	-0.2225
	Eye diameter (mm)	10	*1.486e-13*	0.2724
		12	*8.620e-13*	0.2654
		15	*<2.20e-16*	0.3129
	Age (dpf) at hatch (0 dph)	10	*0.0003*	-0.3039
		12	*8.627e-06*	0.2517
		15	*<2.20e-16*	0.5086

Figure Legends

Figure 1. Longfin smelt larval (0-3 dph) spinal deformities. a) Normally developing larvae (*0*), 2 dph/16 dpf. b) Larvae experiencing first-level (*i*) abdominal spinal kyphosis and first-level tail spinal kyphosis, 3 dph/17 dpf. c) Larvae experiencing second-level (*ii*) post-cranial spinal kyphosis, 2 dph/16 dpf. d) Larvae experiencing third-level (*iii*) "c-shaped" deformity, 2 dph/14 dpf. All larvae pictured here were from the 15℃ treatment group.

Figure 2. Total length (mm) of LFS larvae by temperature treatment (10, 12, or 15℃) and clutch with pooled replicates at each sample date from 0-3 dph. Significance results embedded in the legend (alpha = 0.05). The first letter (a, b) corresponds to differences *within* temperature groups *between* clutches (ie. clutch 1, 2, and 3 at 10℃). The second letter (x, y, z) corresponds to differences *within* clutches *between* temperature groups (ie. clutch 1 at 10, 12 and 15℃).

Figure 3. Frequency of spinal deformity between temperature treatment groups, organized by severity of deformity according to a modified scale from Cobcroft et al. (2004). Clutches were pooled within temperature treatments to show variation. Statistical analyses were not performed on deformity data.

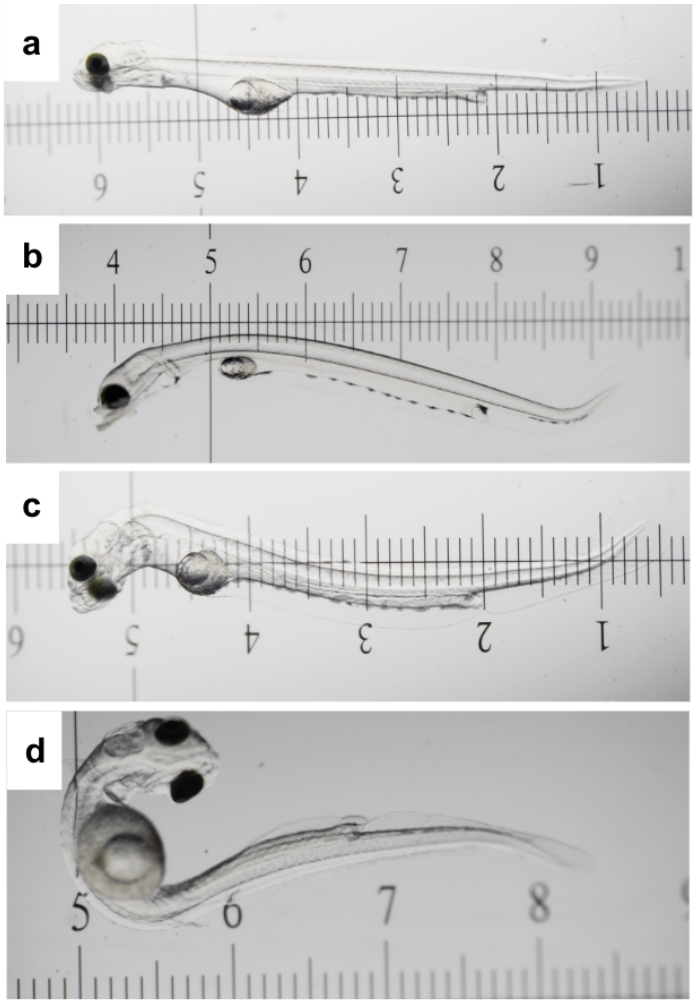


Figure 1

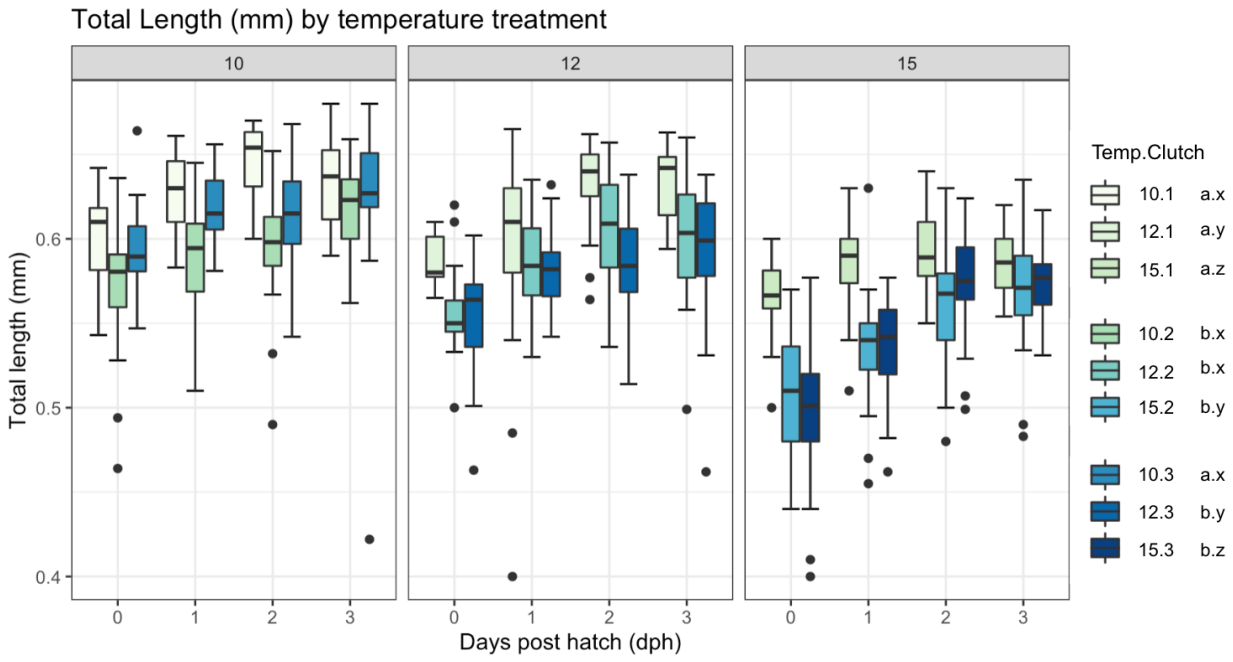

Total Length (mm) by temperature treatment
10
12
15
Total length (mm)
0.6
0.5
0.4
0
1
2
3
Days post hatch (dph)
Temp.Clutch
10.1 a.x
12.1 a.y
15.1 a.z
10.2 b.x
12.2 b.x
15.2 b.y
10.3 a.x
12.3 b.y
15.3 b.z

Figure 2

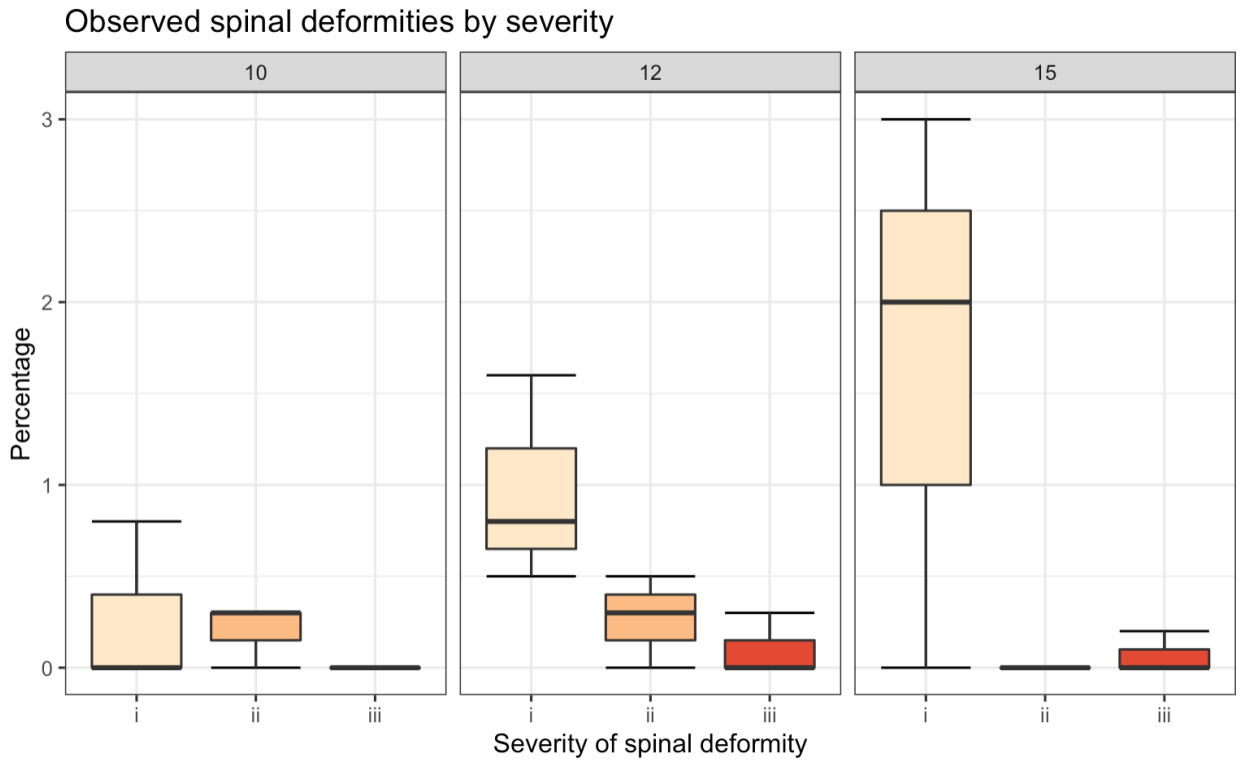
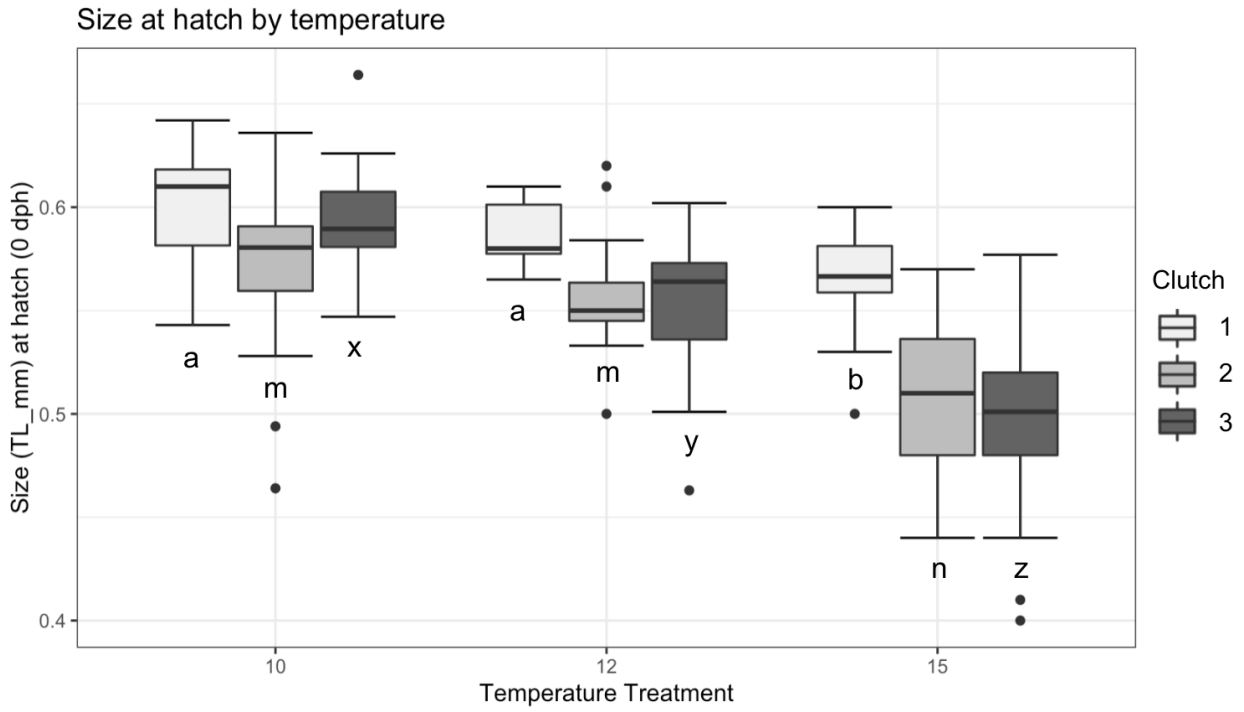

Observed spinal deformities by severity
10
12
15
3
2
1
0
Percentage
i
ii
iii
Severity of spinal deformity

Figure 3

Appendices

Appendix A. Broodstock morphology and clutch size information for the three clutches utilized for the project. Clutch 3 was the only clutch with a 1-2 female-male spawning pair due to low sperm count in the first spawned male. The comma separated values for clutch 3 represent the first and second spawned males, respectively.

Clutch	Spawn date	Female/Male length (mm)	Female/Male mass (g)	Approximate clutch size at spawn	Fertilization success (%)
1	Feb 25, 2022	97/118	7.1/12.3	3700	14.9
2	Feb 25, 2022	109/103	12.2/8.2	6500	13.8
3	Mar 2, 2022	118/89, 109	12.7/6.5, 7.5	2900	27.6



Appendix B. Size (total length, mm) of LFS larvae at hatch (0 dph) presented by temperature treatment. Significance results represented by letters beneath boxes. Comparisons were made

between temperature treatments and within clutches (a, b for clutch 1; m, n for clutch 2; x, y, z for clutch 3).