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Research article

Growth, osmoregulation and ionoregulation of longfin smelt (*Spirinchus thaleichthys*) yolk-sac larvae at different salinities

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Longfin smelt (*Spirinchus thaleichthys*) is a threatened anadromous fish species that spawns in freshwater to moderately brackish (i.e. 5–10 ppt) reaches of the upper San Francisco Estuary and has declined to ∼1% of its pre-1980s abundances. Despite 50+ years of population monitoring, the efficacy of 10+ years of conservation efforts for longfin smelt remain uncertain due to a limited understanding of how the species responds to environmental variation, such as salinity. For example, high mortality during larval stages has prevented culture efforts from closing the life cycle in captivity. Here, we investigated the effects of salinity on longfin smelt yolk-sac larvae. Newly hatched larvae from four single-pair crosses were acutely transferred to and reared at salinities of 0.4, 5, 10, 20 or 32 ppt. We compared whole-body water and sodium ion (Na^+) content, notochord length and yolk-sac volume at 12, 24, 48, 72, and 96 hours post-transfer for each salinity treatment. We found that larvae maintained osmotic and ionic balance at 0.4–10 ppt, whereas salinities >10 ppt resulted in decreased water and increased whole-body Na^+ content. We also found that larvae grew largest and survived the longest when reared at 5 and 10 ppt, respectively, and that yolk resorption stalled at 0.4 ppt. Finally, there were significant but small interclutch variations in responses to different salinities, with clutch accounting for <8% of the variance in our statistical models. Overall, our results indicate that longfin smelt yolk-sac larvae likely perform best at moderately brackish conditions, thus yielding a mechanism that explains their distribution in field surveys and providing key information for future conservation efforts.

Key words: yolk resorption, San Francisco Estuary, fish, early life stages, Aquaculture

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Introduction

Longfin smelt (*Spirinchus thaleichthys*) is a pelagic osmerid forage fish that occupies coastal habitats in the northeast Pacific Ocean, from the Aleutian Islands, Alaska in the north to the San Francisco Estuary (SFE), California in the south (Moyle, 2002; Garwood, 2017; Saglam *et al.*, 2021). This species is described as exhibiting an anadromous life history, where adults migrate from coastal marine habitats in autumn to streams and estuaries to spawn, though some landlocked freshwater populations do exist (Moulton, 1974; Moyle, 2002; Garwood, 2017; Lewis *et al.*, 2019). The SFE supports the southernmost population of longfin smelt and likely serves as a source population for several smaller coastal populations in Northern California and Southern Oregon (Saglam *et al.*, 2021). Longfin smelt were once one of the most abundant fish species in the SFE and likely served an important ecosystem service as a pelagic forage fish; however, the population declined precipitously in the 1980s and is now ∼1% of its historic abundance (Rosenfield and Baxter, 2007; Sommer *et al.*, 2007; Hobbs *et al.*, 2017; Tempel *et al.*, 2021). This elevated risk of extirpation led to their listing as 'threatened' under the California Endangered Species Act in 2009 and the implementation of efforts to improve conservation guidelines to minimize impacts to the species from water project operations in the South Delta (CDFG, 2009; Nobriga and Rosenfield, 2016; Hobbs *et al.*, 2017).

Although anthropogenic, physical and biological changes to the SFE are implicated in the decline of longfin smelt, the primary cause(s) remain uncertain. Diversion of inflowing river water to the estuary has long been implicated for the decline of longfin smelt, as well as many other pelagic fishes in the SFE (Stevens and Miller, 1983; Jassby *et al.*, 1995; Kimmerer, 2002b; Thomson *et al.*, 2010). For example, longfin smelt abundance in the fall has a strong positive correlation with winter–spring freshwater outflow, of which between 35% and 65% can be diverted for the agricultural and urban sectors depending on the water year (Cloern and Jassby, 2012). Freshwater flows mediate many physicochemical variables in the estuarine environment such as temperature, salinity, turbidity and nutrient transport (Kimmerer, 2002a), and the magnitude of outflows dictates the geographic distribution and environmental conditions that larvae experience (Dege and Brown, 2004; MacWilliams *et al.*, 2016; Lewis *et al.*, 2020; Lewis *et al.*, 2019; Grimaldo *et al.*, 2020). In high flow years, young longfin smelt can be found in the western region of the northern estuary (Grimaldo *et al.*, 2020) and southern estuary (Lewis *et al.*, 2020), while in low flow years, they are confined to Suisun Bay and the Delta where fish are entrained into the many pumping facilities of the State Water Project and Central Valley Project (Eakin, 2021; Tempel *et al.*, 2021). Furthermore, the SFE is fraught with numerous invasive species that have altered the estuary's food web (Kimmerer, 2002b; Baerwald *et al.*, 2012; Nobriga *et al.*, 2013; Sabal *et al.*, 2016) and contaminants (Connon *et al.*, 2019; Fong *et al.*, 2016; Weston *et al.*, 2019) that have had negative consequences for other imperilled native fish species such as Chinook salmon (*Oncorhynchus tshawytscha*) (Giroux *et al.*, 2019; Fuller *et al.*, 2021) and delta smelt (*Hypomesus transpacificus*) (Mundy *et al.*, 2020, 2021).

To understand the causes of the decline, conservation managers have intensified efforts to study longfin smelt ecology in order to help inform effective management strategies (Cowin and Bonham, 2013). One important facet of longfin smelt conservation is the development of a fish culture program (CDFW, 2020) to ultimately close the life cycle of longfin smelt in captivity. This captive culture would provide specimens for research and be used for supplementation of wild populations, as necessary. The UC Davis Fish Conservation and Culture Laboratory (FCCL) has been working to develop a captive refuge population of longfin smelt since 2010. However, there has historically been low survival [e.g. ∼2% to 120 days post-hatch (dph) and 0.01% to 300 dph] of cultured fish (Tigan *et al.*, 2019).

Larvae are one of the most sensitive life stages and high mortality during this period acts as the first major bottleneck for the recruitment of a fish species (Hjort, 1914). Thus, determining the mechanisms by which mortality occur could greatly inform conservation management. Identifying some of the environmental correlates of abundance for the larval life stage has been of particular importance for guiding conservation management of longfin smelt (Grimaldo *et al.*, 2017). Temperature, salinity and water clarity have strong correlations with larval abundance, whereby abundance peaks between temperatures of 8°C and 12°C, practical salinities of 2–6 (roughly equivalent to parts per thousand, ppt) and Secchi disc depths of 50 cm (Grimaldo *et al.*, 2017; Hobbs *et al.*, 2010). Defining the environmental correlates for the abundance of longfin smelt has informed hypotheses about the optimal environmental conditions and tolerances for longfin smelt at different life stages.

While environmental correlates of longfin smelt abundance equips conservation managers with an important tool for directing conservation efforts such as habitat restoration and freshwater flow management, it is important to empirically validate field observations and gather more detailed data on the physiological tolerances of species using experimental approaches that explore mechanistic relationships. This is particularly important for conservation aquaculture, where precisely controlled culturing conditions must be employed to optimize production to meet research and population conservation demands such as supplementation. Thus far, temperature effects on longfin smelt larvae have been experimentally evaluated, with 9°C or 12°C being identified as appropriate culturing temperatures for longfin smelt embryos and larvae (Yanagitsuru *et al.*, 2021). These results corroborated findings from field studies, and it is thus unlikely that the temperatures used for culturing longfin smelt at the FCCL (12°C) have limited the success of prior culturing efforts. However, given the strong correlation between larval catches and salinity *in situ*, experimental studies of tolerances of early life stages to

variation in salinity remain a critical next step in developing an effective culture program.

To further inform our understanding of the habitat needs, tolerances and larviculture requirements for longfin smelt, we evaluated the responses of yolk-sac larvae to five salinities (0.4, 5, 10, 20 and 32 ppt), where 0.4–20 ppt encompassed the range of salinities where longfin smelt larvae have been found in the SFE; 5 ppt represented a salinity where larvae are most abundant (2–6 ppt) (Grimaldo *et al.*, 2017), and 32 ppt represented the salinity at the mouth of the San Francisco Bay (Shellenbarger and Schoellhamer, 2011). The responses measured included survival, osmotic and ionic balance, growth and yolk utilization. Given that larval surveys (Grimaldo *et al.*, 2017, 2020; Hobbs *et al.*, 2010) and otolith geochemistry studies (Hobbs *et al.*, 2010; Lewis *et al.*, 2019) have each identified lightly brackish (i.e. 0.5–5 ppt) conditions as important for larval longfin smelt, we predicted that longfin smelt would exhibit optimal physiological responses at 5 ppt. Alternatively, we expected longfin smelt to exhibit optimal physiological responses at 10 ppt as this salinity is approximately isosmotic to the internal osmolality of fish and would thus confer reduced energetic costs to osmotic and ionic regulation. Results of this work are key for informing population models, habitat restoration and captive culture of this threatened estuarine species.

Methods

Broodstock and embryo collection

Adult broodstock longfin smelt were collected from the wild by the U.S. Fish and Wildlife Services Chipps Island trawl with a midwater trawl, the FCCL using a lampara net in the Sacramento San Joaquin Delta and the UC Davis Otolith Geochemistry and Fish Ecology Laboratory using an otter trawl in the Alviso Marsh in South San Francisco Bay. Broodstock collections were approved under California Department of Fish and Wildlife MOU ID: Hobbs_LFS_2021 and Specific Use Permit IDs S-191990002-19, 199-001 and D-0021521915-6. Fish were held in 76 l cylindrical carboys during collections and transported to the FCCL at the end of each sampling day and held at 10 ppt at 12°C until visually gravid and then strip-spawned. A total of four clutches from separate single-pair crosses were obtained between January and April 2019 (see Supplementary Table S1 for parental information), and embryos were maintained using methods described in Yanagitsuru *et al.* (2021). Fertilized embryos were held at 12°C in pre-treated Delta water (0.2 ppt; see Supplementary Table S2 for additional water constituents) at the FCCL until 5 or 7 days post-fertilization (dpf) when fertilized and unfertilized embryos could be differentiated.

Fertilized embryos were transported to UC Davis fish conservation physiology laboratory in conical tubes within coolers chilled with two ice packs. The ice packs were separated from direct contact with the tubes with a Styrofoam pad to prevent temperatures from cooling too much. By the time embryos arrived, water temperatures within tubes were between 11.7 and 13.2°C. Upon arrival, embryos were placed in 500 ml nylon mesh (mesh size, 100 μm) containers within gently aerated 2-gal buckets receiving flow-through ($\sim$4 l h^{-1}) freshwater (well water; 0.4 ppt; see Supplementary Table S2 for additional water constituents). Temperature was lowered gradually to 9°C at 0.5°C h^{-1} as this temperature yielded high hatching success in a previous study (Yanagitsuru *et al.*, 2021). Embryos were exposed to a 12:12 light:dark cycle. Embryos from all clutches were maintained in these conditions and checked twice daily between 8:00–9:00 and 22:00–24:00 to remove any dead embryos (indicated by a milky opaque coloration, similar to delta smelt; Tsai *et al.*, 2021), until hatching. Temperature (8.3–9.2°C), dissolved oxygen (94.8–99.2%), salinity (remained constant at 0.4 ppt) and pH were measured daily with a YSI 556 (YSI, Inc., Yellow Springs, OH, USA). Ammonia (total ammonia nitrogen) was also measured daily with a salicylate colorimetric water test kit (API, Calfont, PA, USA) and remained below detection threshold ($<$0.25 ppm) throughout the duration of the experiment (see Supplementary Table S3 for water parameters). All clutches were monitored for hatching at 8:00 and between 22:00 and 24:00. No hatching was observed between 22:00 and 24:00, indicating that all hatching occurred between 24:00 and 8:00, and thus all individuals used for experimentation were between 0 and 10 hours post-hatch (hph).

Salinity exposures

Clutch 1 was used to measure survival over time at five different salinities: 0.4 (freshwater), 5, 10, 20 and 32 ppt. Water of different salinities were made by mixing well water with Red Sea Coral Pro salt mix (Red Sea Fish Pharm, Ltd, Herzliya, Israel) and stored in heavily aerated and mixed 300 l reservoirs. Between 8:00 and 9:00, hatched larvae were divided into five groups of 30 individuals and acutely transferred to 500 ml mesh containers in separate, gently aerated 2-gal buckets receiving flow-through water ($\sim$4 L h^{-1}) of one of the five experimental salinities. Because yolk-sac larvae do not feed exogenously and to ensure water quality issues from excess feed did not affect survival, larvae were left unfed. Larvae were monitored every 24 hours thereafter, and dead larvae were recorded and removed to determine survival until 192 hours post-transfer (hpt).

Clutches 2, 3 and 4 were used to measure wet and dry mass (used to calculate whole-body water content), notochord length, yolk-sac volume and whole-body sodium ion (Na^+) content over time post-transfer at the five experimental salinities (methods described below). For each clutch, a subset of hatched larvae was collected between 8:00 and 9:00 to gather pre-transfer measurements. All other larvae were divided into groups of 100 individuals into 500 ml mesh containers within 2-gal buckets of different salinities as described above and left unfed. Because the survival trial indicated that substantial mortality began at 96 hpt and because of the limited avail-

ability of this imperilled species, we only sampled up to 96 hpt. Subsets of larvae from each salinity treatment were subsequently sampled at 12, 24, 48, 72 and 96 hpt to gather post-transfer measurements. Temperature (8.3–9.1°C), dissolved oxygen (93.5–100%), salinity (0.4 ppt: 0.4 ppt; 5 ppt: 5.0–5.7 ppt; 10 ppt: 9.9–10.5 ppt; 20 ppt: 19.9–20.8 ppt; and 32 ppt: 31.6–32.4 ppt), pH (8.2–8.6) and ammonia were also measured at this time (Supplementary Table S3). Ammonia remained below detection threshold throughout the duration of the experiment.

Whole body water content and Na$^+$ content

For each clutch, five groups of 10 larvae each ($n = 15$) were sampled pre-transfer and at 12, 24, 48, 72 and 96 hpt to measure wet and dry masses and further used to calculate whole-body water content. Due to high mortality between 48 and 96 hpt in the 32 ppt treatment group for all clutches, sample sizes were reduced at 72 ($n = 5$) and 96 hpt ($n = 5$) for this group. Likewise, clutches 2 and 3 experienced high mortality in the 20 ppt treatment group before 96 hpt and sample sizes were reduced at 96 hpt ($n = 13$; see Supplementary Table S4 for all sample sizes). Larvae were pooled for each mass measurement to ensure measured masses fell within the working range (minimum load: 10000 μg; repeatability: 50 μg) of the Veritas HPB 625i semi-micro balance (Veritas Technologies LLC, Santa Clara, CA, USA) used. Minimum loads were achieved by measuring larvae within 1.5 ml centrifuge tubes. Upon collection, each group of larvae was euthanized with 0.5 g l^{-1} tricaine methanesulfonate (MS-222) buffered to pH 8.0 with sodium bicarbonate ($NaHCO_3$) in accordance with UC Davis IACUC protocols and rinsed over a 100-μm nylon mesh screen with 1 ml of Millipore water to remove any residual salts on body surfaces. Larvae were gently dried by wicking away water with a KimWipe (Kimberly-Clark Worldwide, Inc., Roswell, GA, USA) and then weighed for wet mass. During preliminary testing, consecutive measures of wet mass were found to decrease with each repeated measure, suggesting that the samples were quickly drying and all wet mass for experimental samples were thus measured only once. This procedure was completed within 2 minutes of euthanizing larvae. The same groups of larvae were then dried in a convection oven at 60°C over 48 hours and weighed for dry mass. We chose 60°C as this fell within the recommended temperature range by Schmidt *et al.* (2013) and was a temperature close to that used by a similar study by Gallagher *et al.* (2013). Dry mass was measured three times for each sample and the average of masses was used for analyses. Wet and dry masses were divided by 10 (number of larvae per group) to calculate average individual larval wet and dry masses. Whole-body water content was calculated as the difference between wet and dry mass and reported as a percentage of wet mass.

The same dried groups of larvae previously used to measure dry mass were then used to measure whole-body Na$^+$ content following modified methods described in Gallagher *et al.* (2013). Larvae were placed in 0.2 ml microcentrifuge tubes and digested in 10× the wet mass of the pooled group in 1 mol l^{-1} nitric acid (V/W) at 65°C for 1 week. Larvae were dissociated with daily mixings with a vortex shaker (Scientific Industries, Inc., Bohemia, NY, USA) to aid digestion. After digestion, tubes were centrifuged to separate the solids and supernatant was analyzed on a Sherwood Scientific M360 flame photometer (Sherwood Scientific Ltd, Cambridge, United Kingdom) and standardized to dry mass for whole-body Na$^+$ content. Whole-body Na$^+$ content values were divided by 10 (number of larvae per group) to calculate an average individual whole-body Na$^+$ content per sample.

Morphometrics

Subsets of larvae (see Supplementary Table S4 for sample sizes) were imaged with a Canon EO6 Rebel T6 (Canon, Tokyo, Japan) mounted on a Leica S8APO stereomicroscope (Leica Microsystems, Chicago, IL, USA) and analyzed for morphometrics with Fiji ImageJ software following methods described in Yanagitsuru *et al.* (2021). Whole-body larval images were taken next to an electron microscopy grid (grid, 200 μm; bar, 50 μm) to provide a scale for calibrating pixels to mm to measure notochord length. Lateral and ventral images of yolk-sacs were all taken at 12.8× magnification and calibrated to a separate image of an electron microscopy grid at the same magnification to measure yolk-sac length, depth and width. Yolk-sac volume was approximated as an ellipsoid ($V = {}^4/_3 \pi abc$, where V is volume, a is half-length, b is half-depth and c is half-width of the yolk-sac).

Statistical analyses

R 3.6.3 (R Core Team, 2013) was used for statistical analyses. Linear models were used to analyze all metrics. Wet mass, dry mass, whole-body water content, whole-body Na$^+$ content, notochord length and yolk-sac volume were each modelled separately as functions of the fixed categorical effects of salinity, time post-transfer, clutch ID and their interactions. Whole-body Na$^+$ content was log-transformed to meet the assumptions of normality. Tukey's multiple comparison post-hoc tests were used to examine differences between salinities and times post-transfer. Statistical significance was accepted at $P < 0.05$. All values are reported as mean ± SEM unless otherwise stated.

Results

Survival

Larvae experienced the earliest and highest mortality at 32 ppt. The first mortality incidence occurred at 72 hpt, and by 192 hpt, all individuals had perished. Larvae experienced first incidence of mortality at 96 hpt in freshwater and 20 ppt and approximately half of the larvae perished by 192 hpt. Larvae in 5 and 10 ppt had the lowest incidence of mortality out of experimental salinities; only 1 out of the 30 individuals

Table 1: Percent unfed yolk-sac larvae survival over time in different salinities (each salinity group consisted of 30 individuals from clutch 1 at the start of trials)

Hours post-transfer	Salinity (ppt)				
	0.4	5	10	20	32
0	100	100	100	100	100
24	100	100	100	100	100
48	100	100	100	100	100
72	100	100	100	100	90
96	90	100	100	96.7	50
120	83.3	100	100	96.7	33.3
144	70	100	100	56.7	3.3
168	63.3	100	100	56.7	3.3
192	40	96.7	96.7	53.3	0

in each of the 5 and 10 ppt treatments had perished by 192 hpt (Table 1).

Due to subsets of individuals being sampled over time for clutches 2–4, we could not fully assess differences in survival for these clutches. However, we observed differences in mortality at different salinities between clutches that are worth noting. In freshwater, clutch 2 had ~40% of individuals survive by 96 hpt while clutches 3 and 4 had minimal mortality. At 20 ppt, clutches 2 and 3 had ~40% of individuals surviving by 96 hpt. Finally, for 32 ppt, all individuals from clutch 3 perished after 48 hpt while ~20% and 30% of clutches 2 and 4, respectively, remained after 96 hpt. While some discrepancies exist between these observations and the survival measured in clutch 1, the general pattern whereby yolk-sac larvae experience earlier mortality in freshwater, 20 and 32 ppt were consistent.

Wet mass, dry mass and whole-body water content

There was a significant interclutch difference in longfin smelt osmotic balance parameters at different salinities (Table 2). These differences were small, with the interaction of salinity and clutch accounting for 4.0% of the variance in wet mass, 5.4% in dry mass and 1.4% in whole-body water content observed in our model. However, differential survival at the different salinities (described in 'Survival' above) were noteworthy and there were observed differences in the pattern of osmotic balance between clutches (Supplementary Table S4). There were no differences in dry mass between salinities at any timepoint for clutches 3 and 4. Dry mass had minor differences at 12 hpt in clutch 2 whereby larvae in 10 ppt had higher dry mass compared to those in 32 ppt but there were no other differences between salinities at any other timepoint. As a result, differences in whole-body water content were driven largely by differences in wet mass of larvae and these two metrics followed similar patterns. Wet mass increased in and whole-body water content remained stable in 0.4, 5 and 10 ppt for all clutches. For 20 ppt, only clutch 2 had larval wet mass continue to increase by 96 hpt and whole-body water content remained stable throughout the experimental period. For clutches 3 and 4, wet mass did not increase over time and larval whole-body water content was transiently lower at 20 ppt compared to the lower salinities at 72 hpt. In 32 ppt, larval wet mass and whole-body water content were lowest by 24 hpt in clutch 2, 48 hpt in clutch 3 and 72 hpt in clutch 4.

Averaged across all clutches, larval wet mass increased over time in the freshwater, 5 and 10 ppt exposures (Fig. 1A). For 20 ppt, larval wet mass did not increase after 24 hpt and was similar to 32 ppt and significantly lower than 0.4, 5 and 10 ppt by 96 hpt. Larval wet mass in 32 ppt decreased until 72 hpt. There was high mortality after 48 hpt at 32 ppt resulting in reduced sample sizes at 72 and 96 hpt at this salinity. There was a measured increase in wet mass from 72 to 96 hpt in 32 ppt likely due to mortality of all individuals unable to tolerate the acute 32 ppt exposure and only individuals that were tolerant of this salinity being measured. Dry mass did not differ between salinities at any timepoint between salinities (Fig. 1B). We expected that dry mass would decrease over time due to the catabolism of the yolk in unfed larvae. However, it is likely that due to the small size of yolk in longfin smelt, the scale was not sensitive enough to measure any decrease in dry mass. Whole-body water content remained constant over time for freshwater, 5 and 10 ppt treatments (Fig. 1C). Larvae in 20 ppt had a slightly reduced whole-body water content compared to the lower salinities, and larvae in 32 ppt had highly reduced whole-body water content compared to all other salinities between 24 and 72 hpt. There was a measured increase in whole-body water content between 72 and 96 hpt at 32 ppt this was likely due to only surviving individuals tolerant of this salinity being measured; there was high mortality that may have resulted in selection of salinity-tolerant individuals by this timepoint.

Whole-body Na^+ content

There was a significant interclutch difference in whole-body Na^+ content for longfin smelt yolk-sac larvae at different salinities that accounted for 7.5% of the variance in our model (Table 2). In clutch 2, larvae in 20 ppt did not have any differences in whole-body Na^+ content from the lower salinities. In clutch 3, whole-body Na^+ content stabilized at a higher level from pre-exposure by 72 hpt. In clutch 4, whole-body Na^+ content continually increased for the duration of the exposure. Averaged across all clutches, whole-body Na^+ content remained unchanged at freshwater and 5 ppt (Fig. 2). There were no significant differences in whole-body Na^+ content at any timepoint at 10 ppt compared to freshwater and 5 ppt but there was a trend whereby Na^+ content stabilized at higher levels between 72 and 96 hpt. At

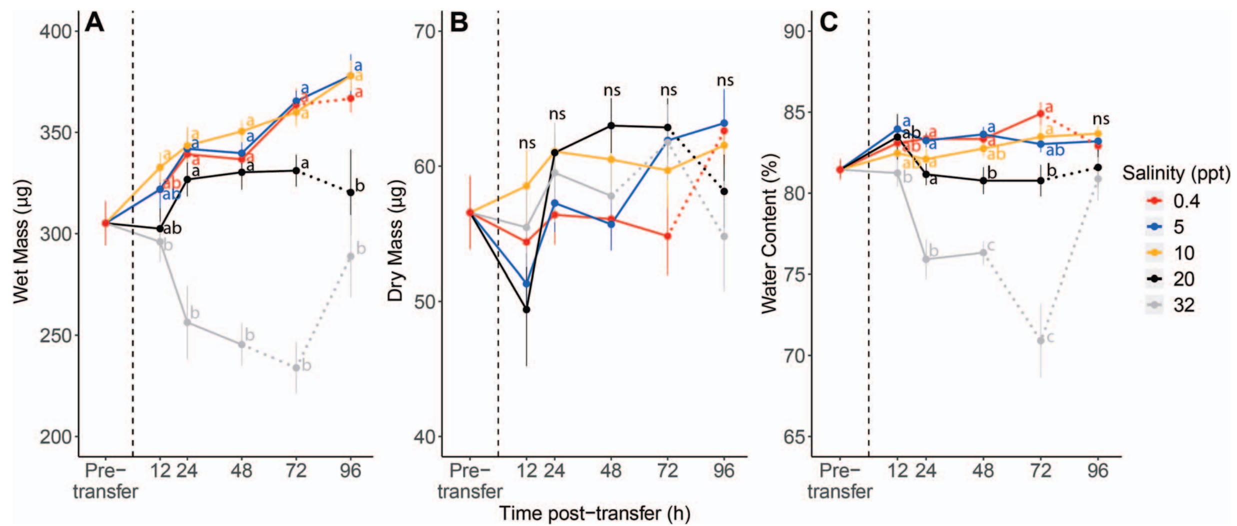

Figure 1: Wet mass (**A**), dry mass (**B**) and whole-body water content (**C**) of longfin smelt yolk-sac larvae after transfer to different salinities. Vertical dashed lines separate pre- and post-transfer timepoints. Dotted lines indicate reduced sample size due to mortality (see Supplementary Table S4 for sample sizes). Letters denote significant differences ($P < 0.05$).

20 and 32 ppt, there was elevated whole-body Na^+ content by 48 and 12 hpt, respectively.

Notochord length and yolk-sac volume

There were significant interclutch differences in notochord length and yolk-sac volume at different salinities, but these effects were small; the interaction between salinity and clutch accounted for only 3.3% and 0.6% of the variance observed in our models for notochord length and yolk-sac volume, respectively (Table 2). For clutch 2, larvae in 5 and 10 ppt had higher notochord lengths than all other larvae by 96 hpt. But for larvae in clutch 3, notochord lengths were similar in freshwater, 5 and 10 ppt by 96 hpt. Finally, larvae in clutch 4 had highest notochord lengths at 5 ppt by 96 hpt. Averaged across all clutches, larvae reared at 5 and 10 ppt had the highest notochord length by 96 hpt (Fig. 3A). Notochord lengths of larvae reared at 32 ppt decreased by 12 and 24 hpt but recovered by 96 hpt, but this is likely due to only surviving individuals being measured. Yolk-sac volume decreased at the same rate over time in all salinities except for freshwater, where yolk resorption was stalled between 12 and 48 hpt and between 72 and 96 hpt, resulting in slower yolk resorption.

Discussion

We demonstrated experimentally that longfin smelt yolk-sac larvae in the SFE perform best in moderately brackish conditions (i.e. 5–10 ppt) that occur downstream of tidal freshwater habitats. These results have implications for informing culturing methods and life cycle models for longfin smelt. For example, our results suggest that the life histories of longfin smelt are more complex than simple anadromy (spawning in freshwater followed by migration to the ocean), with optimal conditions for spawning or rearing possibly occurring in moderately brackish estuarine habitats. Similarly, our results suggest that longfin smelt is an obligate freshwater-estuarine spawner that is unlikely to utilize marine-dominated habitats for reproduction, contrasting with its coastal marine sister species, the night smelt (*Spirinchus starski*) (Love, 2011). Furthermore, our laboratory results are corroborated by salinity reconstructions using otolith strontium isotope geochemistry on field-sampled fish (Hobbs *et al.*, 2010; Lewis *et al.*, 2019) and the distribution of larvae in relation to salinity gradients (Grimaldo *et al.*, 2017, 2020; Hobbs *et al.*, 2010). By identifying the physiological foundations for early reliance on moderately brackish habitats, we are able to better inform future efforts to develop an effective conservation culture program for longfin smelt, to model its population dynamics and restore critical habitats for this threatened estuarine species.

Survival of yolk-sac larvae was highest (96% each) in the 5 ppt and 10 ppt treatments, adding evidence that moderately brackish habitats provide optimal rearing conditions for SFE longfin smelt. These results are consistent with prior results using otolith geochemistry to examine the relative survival of wild fish that reared in various salinity conditions (Hobbs *et al.*, 2010). Furthermore, larvae reared at 5 ppt in all clutches grew to become among the largest out of all treatments, further indicating that moderately brackish habitats are preferential for larval longfin smelt. The hypothesis that longfin smelt larvae perform better in moderately brackish conditions upon hatch is further supported by the minimal disruption of whole-body water and Na^+ content for larvae exposed to salinities between 0.4 and 10 ppt.

Table 2: Results of linear models analyzing longfin smelt salinity tolerance metrics for clutches 2–4

Wet mass	SS	Df	F	*P*-value
Salinity	276 446	4	84.12	<0.001*
Hours post-transfer	64 634	5	15.73	<0.001*
Clutch ID	20 406	2	12.42	<0.001*
Salinity × hours post-transfer	61 390	16	4.67	<0.001*
Salinity × clutch ID	32 817	8	4.99	<0.001*
Hours post-transfer × clutch ID	91 018	10	11.08	<0.001*
Salinity × hours post-transfer × clutch ID	42 871	29	1.80	0.009*
Residuals	231 693	282		
Dry mass	SS	Df	F	*P*-value
Salinity	686.3	4	2.56	0.039*
Hours post-transfer	1630.9	5	4.87	<0.001*
Clutch ID	1847.5	2	13.80	<0.001*
Salinity × hours post-transfer	1676.4	16	1.57	0.078
Salinity × clutch ID	1603.3	8	2.99	0.003*
Hours post-transfer × clutch ID	1891.4	10	2.83	0.002*
Salinity × hours post-transfer × clutch ID	1363.5	29	0.70	0.874
Residuals	18879.3	282		
Whole-body water content	SS	Df	F	*P*-value
Salinity	0.126	4	51.25	<0.001*
Hours post-transfer	0.015	5	4.95	<0.001*
Clutch ID	0.006	2	5.24	0.006*
Salinity × hours post-transfer	0.046	16	4.71	<0.001*
Salinity × clutch ID	0.006	8	1.20	0.297
Hours post-transfer × clutch ID	0.025	10	4.11	<0.001*
Salinity × hours post-transfer × clutch ID	0.022	29	1.23	0.202
Residuals	0.173	282		
Log(whole-body Na^+ content)	SS	Df	F	*P*-value
Salinity	14.76	4	39.52	<0.001*
Hours post-transfer	1.28	5	2.74	0.019*
Clutch ID	1.79	2	9.61	<0.001*
Salinity × hours post-transfer	2.70	16	1.80	0.030*
Salinity × clutch ID	4.23	8	5.67	<0.001*
Hours post-transfer × clutch ID	2.48	10	2.66	0.004*
Salinity × hours post-transfer × clutch ID	3.11	29	1.15	0.275
Residuals	26.33	282		
Notochord length	SS	Df	F	*P*-value
Salinity	20.087	4	30.61	<0.001*
Hours post-transfer	11.922	5	14.53	<0.001*

(Continued)

Table 2: Continued

Wet mass	SS	Df	F	*P*-value
Clutch ID	15.261	2	46.51	<0.001*
Salinity × hours post-transfer	14.694	19	4.71	<0.001*
Salinity × clutch ID	8.313	8	6.33	<0.001*
Hour post-transfer × clutch ID	10.985	10	6.70	<0.001*
Salinity × hours post-transfer × clutch ID	23.472	36	3.97	<0.001*
Residuals	146.984	896		
Yolk-sac volume	SS	Df	F	*P*-value
Salinity	48 945	4	29.02	<0.001*
Hours post-transfer	193 931	5	91.99	<0.001*
Clutch ID	18 107	2	21.47	<0.001*
Salinity × hours post-transfer	19 723	19	2.46	<0.001*
Salinity × clutch ID	4359	8	1.30	0.243
Hours post-transfer × clutch ID	17 215	10	4.08	<0.001*
Salinity × hours post-transfer × clutch ID	27 940	36	1.84	0.002*
Residuals	381 166	904		

*$P < 0.05$.

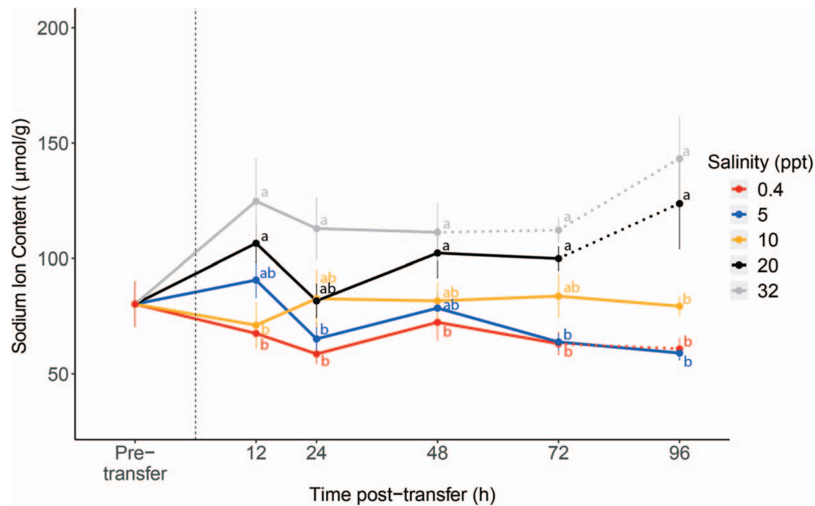

Figure 2: Whole-body sodium ion content of longfin smelt larvae after transfer to different salinities. The vertical dashed line separates pre- and post-transfer timepoints. Dotted lines indicate reduced sample size due to mortality (see Supplementary Table S4 for sample sizes). Letters denote significant differences ($P < 0.05$).

Salinity can influence yolk utilization by either decreasing growth rate with no changes to yolk resorption rate (Bagarinao, 1986; Peterson *et al.*, 1996), by directly affecting yolk resorption rates or by a combination of both (Swanson, 1996; Leiton *et al.*, 2012). For longfin smelt yolk-sac larvae, yolk utilization was influenced in both ways. At the highest salinities of 20 and 32 ppt, growth was reduced, but yolk resorption remained similar as to moderately brackish conditions. In contrast, yolk resorption and growth were both reduced in freshwater relative to moderately brackish conditions. The mechanism behind this reduction in yolk resorption rate under lower salinities is unclear but has been observed in at least two other marine fishes: milkfish (*Chanos* chanos) (Swanson, 1996) and Senegalese sole (*Solea senegalensis*)

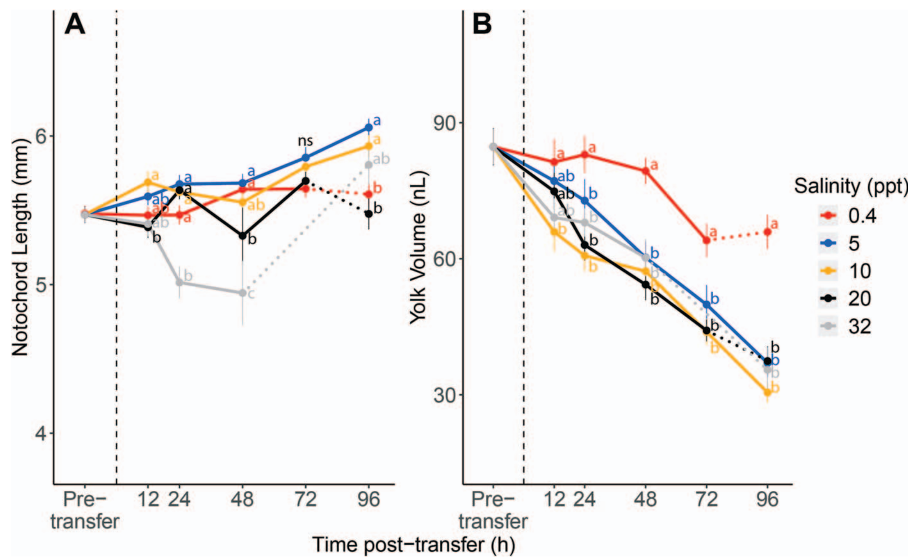

Figure 3: Notochord length (**A**) and yolk-sac volume (**B**) of longfin smelt yolk-sac larvae after transfer to different salinities. Vertical dashed lines separate pre- and post-transfer timepoints. Dotted lines indicate reduced sample size due to mortality (see Supplementary Table S4 for sample sizes). Letters denote significant differences ($P < 0.05$).

(Leiton *et al*., 2012). Increased water content due to osmotic disequilibrium in the yolk-sac could lead to such reductions in yolk resorption (Lein *et al*., 1997; Shi *et al*., 2008). However, our whole-body water content results indicate that osmotic disequilibrium is unlikely to have led to an inflation of yolk-sac volumes. Instead, we suggest that there may be differential and inefficient mobilization of yolk resources when longfin smelt larvae are reared in freshwater. Regardless of the exact mechanism, paired with our observations that longfin smelt yolk-sac larvae experienced mortality before complete yolk resorption, our results suggest that freshwater environments may impede the ability of longfin smelt yolk-sac larvae to resorb yolk and provide some insight into issues with previous culturing attempts and the observed reduction in larval abundance within the SFE below 2 ppt (Grimaldo *et al*., 2017).

Although there were few statistically significant differences in whole-body Na^+ content for larvae exposed to 10 and 20 ppt, it is notable that whole-body Na^+ content for larvae in these salinities stabilized at a higher level compared with the lower salinities with little reduction in whole-body water content. The increase in whole-body Na^+ content could indicate a failure to regulate ionic content within the bodies of longfin smelt larvae. In the case of larvae in 20 ppt, this may be true as there was lower survival and whole-body water content at this salinity compared with the lower salinity treatments. However, the relatively low disruption to whole-body water content and high survival at 10 ppt may suggest that the increase in whole-body Na^+ content could serve an adaptive function similar to that observed in pink salmon (*Oncorhynchus gorbuscha*), another anadromous fish species (Gallagher *et al*., 2013). Increased whole-body Na^+ content would reduce the osmotic gradient between the environment and blood, which would result in lower water loss in hypersaline environments without the potentially high energetic cost of reorganizing cellular structures of ionocytes to maintain osmotic balance (Hiroi *et al*., 2005; Tseng and Hwang, 2008; Ouattara *et al*., 2009; Hsu *et al*., 2014). This may be adaptive for species that hatch in dynamic habitats such as the SFE, where larvae can be rapidly dispersed across strong salinity gradients by large tides, freshwater outflow events and strong winds (Kimmerer *et al*., 2014; MacWilliams *et al*., 2016). In pink salmon, whole-body Na^+ content increases over time even without acclimation to elevated salinities, termed 'precocious anadromy,' which may prepare them for seaward migration (Gallagher *et al*., 2013; Sackville *et al*., 2012). We did not measure an increase in whole-body Na^+ content independent of treatment groups over time in our study. However, this may be due to the short timescale of our experiment and future studies measuring whole-body Na^+ content over a longer timeframe could determine if longfin smelt also exhibit precocious anadromy.

In this study, we focused on yolk-sac larvae, which rely exclusively on their yolk for nutrition, but there may be indirect benefits that moderately brackish conditions may confer for longfin smelt larvae in culture once they begin feeding. For example, many fish larvae in culture, including longfin smelt, require live zooplankton feed. Rotifers (*Brachionus* spp.) and freshly hatched *Artemia* spp. nauplii are common zooplankton used as larval feed in many finfish cultures, including the FCCL (Baskerville-Bridges *et al*., 2005). Rotifers and *Artemia* are reared and hatched in 4–35 ppt (Lubzens, 1987) and 20–30 ppt (Jennings and Whitaker, 1941), respectively, and osmotic shock in rotifers and artemia can cause the zooplankton to sink or perish rather than remain suspended

in the water column. This could reduce their effectiveness as feed in lower salinity cultures for pelagic larvae (Lim *et al.*, 2003) such as longfin smelt. Thus, culturing longfin smelt in higher salinities could have an indirect benefit for larvae by altering the effective availability of live zooplankton feed. This can be confirmed with future studies designed to investigate longfin smelt feeding performance at different salinities.

As expected, most longfin smelt yolk-sac larvae could not tolerate seawater (i.e. 32 ppt), and it is unlikely that longfin smelt larvae would occupy or survive in coastal, marine-dominated habitats upon hatch; however, we also observed notable interclutch variation in tolerance to seawater. For example, half of larvae had survived to 96 hpt in clutch 1, less than one third of larvae in clutches 2 and 4 remained by 96 hpt and all larvae in clutch 3 perished by 72 hpt. Interclutch variation in response to an environmental condition for longfin smelt larvae has also been measured for temperature (Yanagitsuru *et al.*, 2021). While possible that this variation could be explained by genetic differences between clutches or parental effects, clutch accounted for <8% of the variance in our statistical models, suggesting that interclutch variation is relatively small. This is perhaps unsurprising as population genetic studies suggest that the SFE longfin smelt are considered a single intermixing population (Saglam *et al.*, 2021). However, given the large range of survival across clutches, interclutch variation in longfin smelt warrants future investigation through quantitative genetics studies with controlled breeding designs. Until such studies are conducted and given the current results, we instead hypothesize that newly hatched larvae within a clutch of SFE longfin smelt have high interindividual differences in their responses to environmental conditions. High variation in their physiological performance under different conditions could be adaptive in the SFE where physicochemical conditions can vary greatly on tidal, seasonal and interannual time scales. For example, salinity in the SFE can reach salinities as high as 32.5 ppt in some regions (Watson and Byrne, 2009). This variation could thus increase the chances of successful recruitment in an environment where longfin smelt larvae can be found throughout the estuary at different combinations of environmental conditions such as temperature and salinity.

The existence of a few landlocked populations of longfin smelt suggests that the species may be able to complete its lifecycle in freshwater. However, these populations are genetically distinct from coastal populations and are likely locally adapted such that their responses to salinity are different than those of SFE's longfin smelt population. Alternatively, the differences in the balance of different ions of the freshwater that these landlocked populations inhabit may provide physiological benefits that the well water used in the present study did not. Indeed, freshwater is highly variable (up to 1000-fold differences) in composition and can influence fish osmoregulation (Supplementary Table S2; Yanagitsuru *et al.*, 2019; Pinheiro *et al.*, 2021). Nevertheless, it is possible that the SFE population includes individuals with variable physiological tolerances that could allow the population to utilize a variety of different habitat types. This is supported by recent studies of longfin smelt life histories using otolith geochemistry (Lewis *et al.*, 2019). It is believed that managing for the preservation of such diversity can maximize the resilience of migratory populations (Schindler *et al.*, 2010), and this may be particularly important for the SFE's population of longfin smelt given that it functions as a source population for several other coastal populations (Saglam *et al.*, 2021).

Concluding remarks

Here, we demonstrate that newly hatched larval longfin smelt perform best in moderately brackish conditions, with larvae reared in either freshwater or ≥20 ppt salinities exhibiting lower survival, growth and disrupted osmotic and ionic balance. These results challenge both a simple anadromous life cycle and coastal spawning-rearing in marine-dominated habitats and are generally supported by prior studies examining larval distributions *in situ* and otolith geochemistry. Combined with prior results (Yanagitsuru *et al.*, 2021), we recommend the rearing of newly hatched longfin smelt larvae at temperatures of 9–12°C and salinities of 5–10 ppt to maximize growth and survival. However, given that field data suggest that optimal salinities for larval longfin smelt may fall between 2 and 6 ppt (of which lower values were not explored in the present study), future studies examining the effects of lower salinities are likely needed to further refine our understanding of the optimal salinity conditions for larval longfin smelt. Furthermore, studies that investigate the interactive effects of environmental conditions (e.g. temperature and salinity) and ontogenetic changes in physiological responses are likely to shed additional light on the habitat needs of SFE longfin smelt, both in culture and in the wild.

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Author Contributions

Y.R.Y., R.E.C. and N.A.F. conceptualized and designed experiments. Y.R.Y. and I.Y.D. performed experiments and analyzed data. J.A.H. and L.S.L. oversaw collection of broodstock. T.-C.H. oversaw spawning of adult longfin smelt and coordinated provisioning of embryos. Y.R.Y. drafted the orig-

inal manuscript with detailed edits from J.A.H., L.S.L., T.-C.H., R.E.C. and N.A.F. Lastly, Y.R.Y., N.A.F., J.A.H., L.S.L., T.-C.H. and R.E.C. acquired funding.

Data Availability Statement

The data underlying this article are available in Dryad Digital Repository at https://doi.org/10.25338/B84P78.

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Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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