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Author

Hudson, Nicholas

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Evaluating Stress Recovery and Gill Morphology During Experimental Supplementation of an Endangered Fish Species

By

NICHOLAS LUKE HUDSON
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DAVIS

Approved:

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Nann Fangue, Chair

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Amelie Segarra

_____
Brittany Davis

Committee in Charge

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Abstract

      Physiological stress incurred upon transported and released animals may limit the efficacy of population supplementation - a commonly used conservation strategy designed to recover depleted populations. In a recently developed experimental supplementation program for the critically endangered delta smelt (*Hypomesus transpacificus*), combined stressors of handling, transport, and shifts in water chemistry are expected to elicit a considerable stress response. Acclimation enclosures have been developed in an attempt to facilitate stress recovery and allow delta smelt to acclimate to field conditions. Pertinent questions surrounding the use of enclosures include determining the optimal time of release from enclosures and ideal transport densities to minimize stress and maximize fish performance. To address these knowledge gaps, we quantified whole-body cortisol, glucose, lactate, and gill ionocyte morphology at 10 time points across 72 hours following transport to enclosures at high and low densities. We observed significant peaks in cortisol and glucose within 30 min of release into enclosures, followed by significant recovery of both parameters after 27 hours. Lactate was moderately affected, and we did not detect an effect of transport density on cortisol, glucose, or lactate. Gill ionocytes significantly increased in surface area following transport, and we observed gill ionocytes having up to three different types of microvilli, including two types that have never been reported. Changes in ionocyte morphology is correlated to elevated cortisol levels and suggests greater demand for ion-transport at the release site. This work provides agency managers with data to optimize delta smelt supplementation methodologies and expands our understanding of how fishes respond to targeted stressors.

<u>Introduction</u>

The practice of artificially raising and releasing animals into the wild has persisted as a widely employed conservation strategy since the latter part of the 20th century (IUCN 1998, Armstrong and Seddon 2008). These 'supplementation' programs aim to re-establish extirpated species or enhance depleted wild populations and are pervasive across taxa (Kleiman 1989, Welcomme and Bartley 2003, Seddon et al., 2005, Seddon 2007, Griffiths and Pavajeau 2008, Seddon et al., 2014, Machado et al., 2020). The efficacy of supplementation relies upon the robust understanding of species-specific biological and physiological processes that regulate performance and fitness of released individuals (Seddon et al., 2007, Tarszisz et al., 2014, Homberger et al., 2021). One such process that may mediate the success of supplementation programs, especially for highly sensitive species, is the stress response (Teixeira et al., 2007, Dickens et al., 2010, Lamothe et al., 2019). Physiological stressors accompanying the translocation and release of captively raised animals commonly results in an increase of circulating stress hormones (i.e cortisol) and a dysregulation of energy balance (Barton and Iwama 1991, Barton 2000, Hartup et al., 2005, Dickens et al., 2010, Jenni et al., 2014, Batson et al., 2017). These processes, although adaptive, are known to impact health, performance, and fitness if stressors are particularly intense or prolonged (Wendelaar Bonga 1997, Teixeira et al., 2007, Dickens et al., 2010) Although the importance of understanding stress in supplementation is evident, physiological metrics of stress are rarely employed in studies on supplementation methodology. In one review of 120 studies on supplementation, only 9% evaluated traditional physiological metrics, such as the stress response (Tarszisz et al., 2014).

Since the origin of supplementation as a practice, there have been notable successes; the reintroduction of gray wolves (*Canis lupus*) to Yellowstone National Park, USA (Ripple et al.,

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2012), the recovery of the California condor (*Gymnogyps californianus)* in the central California coast, USA (Walters et al., 2010), and the reintroduction of savannah elephants (*Loxodonta africana)* in South Africa (Kuiper et al., 2018). Despite these high profile success stories, many supplementation programs have struggled to quantify success or have failed to reach their goal (Seddon 1999, Pérez et al., 2012, Robert et al., 2015, Taylor et al., 2017). Compared to terrestrial vertebrates, fish have received significantly less attention in the supplementation literature (Seddon et al., 2005), and successes are seen as similarly mixed (Lamothe et al., 2019). Lake sturgeon (*Acipenser fulvescens*) populations in the Great Lakes, USA were recovered following a successful populations supplementation program (Welsh et al., 2020), as were the once extirpated Lahontan cutthroat trout (*Oncorhynchus clarkii henshawi)* in Pyramid Lake, USA (Al-Chockhachy et al., 2019). However, many programs fail to increase population sizes or establish self-sustaining populations (Cochran-Biederman et al., 2015, Sass et al., 2017). In a review of 260 fish supplementation programs, 42% were categorized as unsuccessful (Cochran-Biederman et al., 2015).

For supplemented fishes, stressors include physical handling, confined transport, and rapidly shifting water chemistry (Harmon 2009, Sampaio & Freire 2016). These stressors are known to elicit an activation of the hypothalamus-pituitary-interrenal (HPI) axis and ionic, osmotic, and acid-base (IOA-B) regulatory responses (Barton and Iwama 1991, Barton 2000, Dickens et al., 2010, Sampaio & Freire 2016, Zimmer and Perry 2022). Activation of the HPI axis triggers the release of plasma cortisol, which signals the mobilization of glucose as a rapid source of energy, and is hypothesized to regulate IOA-B responses (Barton and Iwama 1991, Wendelaar Bonga 1997, Barton 2000, 2002, Acerete et al., 2003, Harmon 2009, Kumai et al., 2012). During this process, energy is reallocated away from non-essential processes, and rapid

glucose metabolism provides fuel to starved tissues under increased cardiac output and IOA-B responses needed to regulate blood pH (Wendelaar Bonga 1997, Evans et al., 2005). In O_2-limited tissues under stress, anaerobic glucose metabolism leads to the accumulation of lactate (Milligan and Girard 1993). Although these stress response mechanisms are adaptive and necessary to reestablish homeostasis following stress, chronic or severe stressors can draw energy away from essential processes, deplete energy stores, and reduce overall performance and fitness in the short or long term (Dickens et al., 2010, Schreck 2010, Sokolova 2021). Quantifying species-specific stress responses to supplementation may yield insight into underlying physiological mechanisms limiting the success of fish supplementation programs. However, these studies are rare and often not done in conjunction with active management efforts. Investigating the kinetics of the fish stress response to supplementation could therefore both improve conservation strategies and reveal novel physiological mechanisms through which fish respond to environmental disturbance.

In this study, a recently adopted experimental supplementation program for the critically endangered delta smelt (*Hypomesus transpacificus*) was leveraged to elucidate the magnitude and duration of the stress response to novel supplementation methodology. Delta smelt have been driven to near extinction by an array of disturbances to endemic habitat, the Sacramento-San Joaquin Delta (SSJD, CA, USA), including increased freshwater exports, competition from non-native species, and intense drought (Hobbs et al., 2017, Moyle et al., 2017). Delta smelt were listed as threatened under the California and the Federal Endangered Species Acts in 1993 and were elevated to endangered under the California Endangered Species Act in 2009. In 2012, the species was listed as endangered under the IUCN Red List of Threatened Species. As a safeguard against extinction and to provide individuals for research, the University of California,

Davis Fish Conservation and Culture Laboratory (FCCL; Bryon, CA, USA) has cultured and maintained a refugial delta smelt population since 2007 (Lindberg et al., 2013). In recent years, production of delta smelt at FCCL has accelerated to support an experimental population supplementation program in which cultured adult fish are released to the SSJD. Releases are timed shortly before semelparous reproductive events with the aim that released fish complete a spawning event to support a self-sustaining population. To date, multiple release events have occurred since 2021 resulting in an estimated 190,926 fish released (USFWS 2022, 2023). Release of delta smelt to the SSJD occurs through two methods: "hard release" during which fish are emptied directly from transport vessels into the open water of the SSJD, and "soft release' during which delta smelt are placed into *in-situ* acclimation enclosures designed to incubate them in the SSJD for several days before full release. The use of enclosures has resulted in high survival and positive growth in delta smelt, facilitated acclimatization to natural conditions and transition to wild food sources, and protected delta smelt from initial predation (Buckmeier et al., 2005, Baerwald et al., 2023). Effects of soft release enclosures vary by season and location (Davis et al., in review), and there is very little data on growth and survival for the hard release method. Regardless of methodology, delta smelt supplementation includes an initial period of intense handling followed by prolonged transport and translocation to the SSJD. Following release to the SSJD, adult delta smelt are expected to survive, quickly locate spawning grounds, and reproduce, thereby completing their semelparous one-year life cycle.

The combined stress of handling and two successive rapid changes in water chemistry during supplementation are expected to result in IOA-B challenges for delta smelt. First, initial handling is likely to rapidly increase metabolism thereby increasing blood pCO_2 and H^+, and decreasing blood pH leading to metabolic acidosis (Wells et al., 1986, Ferguson and Tufts 1992,

Cicia et al., 2012, Davison et al., 2023). Prior to transport, transport water is hyperoxygenated and supplemented with increased salinity of ~5 ppt (Swanson et al., 1996), of which the elevated counterions (Na^+ and Cl^-) would presumably boost IOA-B regulation. During transport, respiration likely consumes O_2 and produces CO_2, decreasing pH levels and inducing respiratory acidosis (Paterson et al., 2003, Sampaio & Freire 2016). Finally, upon release to the atmospheric-equilibrated and less saline SSJD (~0.1 ppt), delta smelt would experience yet another IOA-B disturbance, but must continue to regulate blood pH and recover from handling and transport stress without the aid of hyperoxygenation and higher salinity levels.

Acidosis recovery in freshwater fish involves the removal of excess H^+ in exchange for environmental Na^+ to restore blood pH and the accumulation of HCO_3^- to buffer further pH changes (Evans et al., 2005, Hwang et al., 2011, Glover et al., 2013, Tresguerres et al., 2023). As such, delta smelt are likely actively exchanging Na^+ for H^+ and accumulating HCO_3^- to mitigate acidosis during transport (Sampaio & Freire 2016). However, upon their release into the atmospheric-equilibrated SSJD, delta smelt likely experience a rapid increase in blood pH inducing respiratory alkalosis and must offload accumulated HCO_3^- (Lonthair et al., 2020). The compensatory IOA-B response to alkalosis in freshwater fish is similarly well studied and involves the exchange of environmental Cl^- with HCO_3^- to restore pH levels (Evans et al., 2005, Hwang et al., 2011, Glover et al., 2013, Tresguerres et al., 2023).

Ionocytes (formerly called chloride cell and mitochondria-rich cell) are specialized cells responsible for IOA-B regulation and are found on the skin of larval fishes (Hwang et al., 2011, Glover et al., 2013) and the gills of adult fishes (Evans et al., 2005, Tresguerres et al., 2023). Ionocyte responses are dynamic, and one mechanism to facilitate greater ion-transport is by altering the apical morphology of the cell (Kwan et al., in review). The apical surface of gill

ionocytes can be rapidly expanded and microvilli extended within 2-6 hours (Goss et al., 1992, 1994, Montgomery et al. 2022) to increase surface area and facilitate ion-exchange with the environment (Evans et al., 2005). Moreover, abundance and surface area of ionocytes is known to increase with stress response mechanisms such as increased cortisol levels (Laurent and Perry 1990, Goss et al., 2001, Evans et al., 2005, Brannen and Gilmour 2018).

The series of stressors facing delta smelt during supplementation are likely to result in a multi-faceted stress response that could limit the performance of released individuals, and soft release may offer a sanctuary for recovery. However, the optimal duration needed for recovery and acclimation in enclosures prior to full release is not well understood. Neither is the optimal density at which to transport delta smelt to minimize the stress response (Ruane et al., 2002, Liu et al. 2016). In this study, we conducted a field experiment that leveraged and replicated agency delta smelt pilot release efforts (i.e supplementation) to quantify the magnitude and duration of their stress response when using the soft release methodology at two density levels. We monitored water chemistry throughout a ~2 hour transport period and quantified trends in cortisol, glucose, lactate, and latent mortality following release into enclosures in the SSJD at 10 timepoints across 72 hours. We also measured trends in gill ionocyte surface area (SA) in a subset of time points to quantify IOA-B response to shifting water chemistry across transport and after release to enclosures. Our study aims to 1) inform ongoing delta smelt supplementation efforts by quantifying an appropriate acclimation period after which stress levels are minimized, determining if transport densities can be increased, and providing a stress-response baseline for future release strategies, and to 2) contribute to the literature on HPI axis and IOA-B stress response that can inform how fishes respond to targeted stressors.

Materials and Methods

Enclosure Design and Deployment

Cylindrical enclosures (height: 1.2 m, diameter: 1 m) were constructed out of aluminum painted black with mesh size optimal for passage of delta smelt food items and exclusion of predators (detailed in Baerwald et al., 2023). Eight enclosures were deployed in the SSJD at the city of Rio Vista, CA, USA (38.1439, -121.6931) on November 9th, 2022. Enclosures were connected in a line using steel cable and anchored in place by pylons (Figure 1). Orientation and anchoring of the enclosures ensured minimal movement during tidal fluxes, and no contact with other stationary objects in the SSJD. One additional enclosure was deployed within a large recirculating tank (height: 1.5 m, width 20 m) at FCCL as a comparison to conditions in the SSJD (Figure 1). Buoys connected to the rim of all enclosures maintained positive buoyancy with limited head space and allowed ease of access to the interior of the enclosures via secured lids. Enclosures were deployed three weeks before the experiment began to ensure secure placement and anchoring through weather events.

Field Experiment and Water Quality Measurements

Delta smelt were fasted for 24 hours prior to the experiment. Salinity was maintained at 0.3 ppt in FCCL rearing tanks and measured using a YSI PRO2030 (Xylem Incorporated, Washington DC, USA). On the day of the experiment, carboy water was hyperoxygenated to ~20 mgL^{-1} and salted to ~5 ppt with Instant Ocean (Spectrum Brands Family, Blacksburg, VA, USA). Before being loaded with fish, carboy water (75 liters) was then measured for temperature, dissolved O_2, and pH with a multiparameter YSI (650 MDS; Xylem Incorporated, Washington DC, USA). Alkalinity was measured in six of the nine carboys with a digital titrator (Hach AL-DT ; Hach Company, Loveland, CO, USA). Then, 2200 juvenile delta smelt (200

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days post hatch, 69.0 ± 6.0 mm fork length, 2.5 ± 0.8 g; mean $\pm$ s.e.m) were netted from holding tanks into the nine carboys at densities of 200 or 300 fish per carboy (~2.6 or 4 fish per liter) and carboys were sealed with an airtight lid. Eight carboys were transported by truck from FCCL to the SSJD (hereafter called 'field') and loaded onto boats and ferried to the enclosures. It took ~2 hours from when carboys were sealed to when they arrived at the field enclosures. An additional carboy at a density of 200 fish was transported by truck for an equal duration and moved alongside an outdoor tank at FCCL (hereafter called 'lab'). Temperature, dissolved O_2, pH, and alkalinity were measured again in carboys after transport to the field and lab locations. Partial pressure of carbon dioxide (pCO_2) in carboys before and after transport was calculated with CO2SYS (Lewis and Wallace 1998) using measured pH and alkalinity values. Data obtained from a United States Geological Survey Monitoring Station (Station #11455385) ~5 kilometers upstream of the release location measured salinity at 0.1 ppt when fish were released into enclosures. One day prior to transport and release into enclosures, 20 delta smelt were removed from their rearing tanks at FCCL, euthanized with buffered MS-222 (0.5 gL^{-1}; Sigma-Aldrich, St. Louis, MO) and immediately frozen on dry ice to serve as a control for (hereafter called "control").

Starting at 11:20 AM on November 29th, 2022, fish were released from carboys into the eight field enclosures at a density of 200 or 300 fish per enclosure, and into the single lab enclosure at a density of 200 fish (Figure 1). As each carboy was emptied into an enclosure, eight fish were immediately sub-sampled and euthanized. Five fish were measured for length (mm) and snap-frozen on dry ice, and stored at -80°C for later cortisol, glucose, and lactate analysis. The remaining three fish were fixed in 10% formalin (C993M42; Thomas Scientific, Swedesboro, NJ, USA) and stored at room temperature for gill ionocyte imaging. Sampling was

repeated at each enclosure at 0.5, 1, 3, 6, 20, and 27 hours following the release of fish (Figure 1), and at each time took no more than 5 min to complete. Sampling at each field enclosure was staggered to ensure consistency of time points between enclosures. This procedure resulted in a sample size of 20 field fish and 5 lab fish per time point per density treatment for cortisol and metabolites. A total of 12 field fish and 3 lab fish per time point per density treatment were sampled for gill ionocytes. Visible (floating) mortalities were counted in each enclosure at each time point and removed when possible without disrupting other fish, and all non-visible mortalities (submerged) were counted at the end of the experiment.

High winds forecasted for the evening following the 27 hour time point were predicted to cause significant wave actions within the enclosures, potentially leading to high mortality. In order to maintain a reliable count of non-visible mortalities in the enclosures due to transport stress, two enclosures from each density treatment (four enclosures total) were removed from the field following the 27-hour sampling time point, mortalities were counted, and remaining fish were euthanized. Following the wind event, sub-sampling continued at the remaining four enclosures in the field and the enclosure at the lab at 48, 52, and 72 hours (Figure 1). This resulted in a halved sample size at each time point following the 27 hour time point. After sampling at the 72 hour time point, all remaining enclosures were removed from the field and lab site, mortalities were counted and remaining fish were euthanized and measured for length and weights. All fish were collected under IACUC protocol #23829.
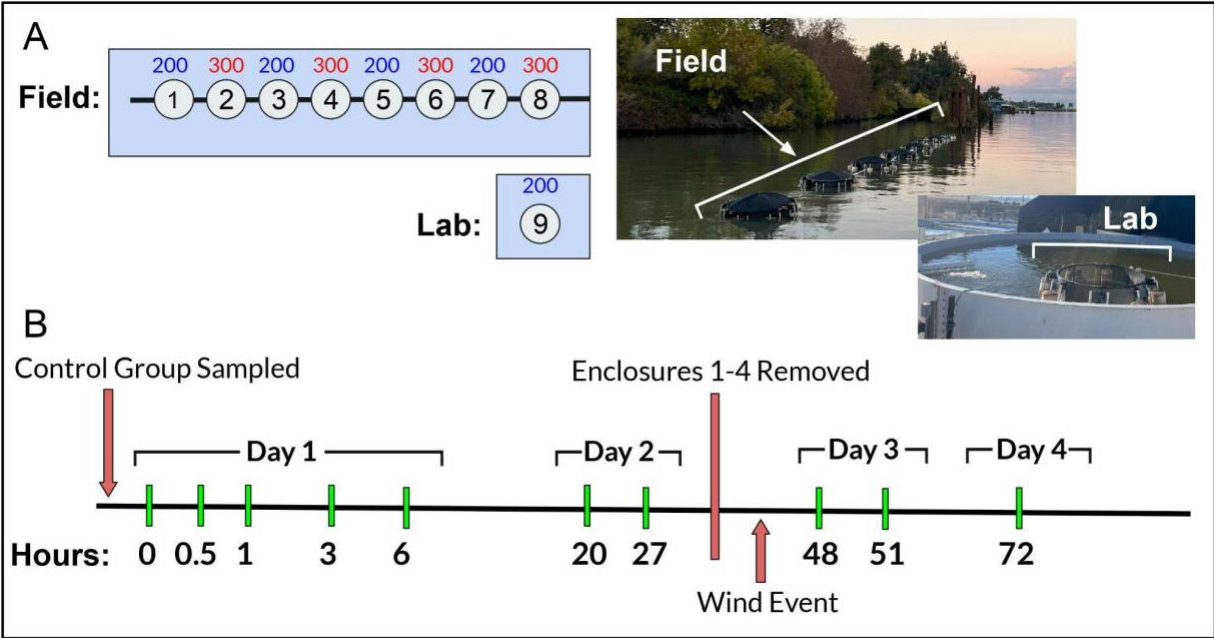
Figure 1: Field and lab enclosure design and deployment location including density treatments (A) and sampling timeline schematic and point of enclosure removal (B). White brackets and arrows indicate enclosure placement in images. Enclosures in the field ("field") are numbered 1-8, and the lab enclosure ("lab") is numbered 9. Blue and red numbers represent 200 (low density) or 300 (high density) of fish per enclosure. Red lines and arrows denote the sampling point for the control group ("control"), and when enclosures were removed and when the wind event occurred. Green lines denote fish sampling timepoints.

Sample Preparation

Whole-body homogenization of delta smelt was used for cortisol, glucose, and lactate extraction following methods outlined in Pasparakis et al. (2022). Fish heads were removed to avoid confounding effects on cortisol and metabolite values before remaining whole bodies were ground into a fine powder over liquid nitrogen using a mortar and pestle. Ground powder was subdivided into two tubes for separate cortisol and metabolite (glucose and lactate) extraction. All powder samples were weighed to be within the range of 100-130 mg. Twelve randomly selected fish of the 20 collected per time point per density treatment were used in cortisol and metabolite analysis for the field location. All 5 fish collected at each time point at the lab were analyzed.

Cortisol Extraction and Assays

Cortisol extraction and assays followed methods in Hasenbein et al. (2013) and Pasparakis et al. (2022). Powdered fish tissue was homogenized with 1 mL of 1x phosphate buffered saline (PBS ; P5368, Sigma-Aldrich, St. Louis, MO) in a polytron-type homogenizer (PowerGen 125, Fisher Scientific, Hampton, NH, USA) over ice for 15 sec. Homogenates were then spiked with 2.5 mL of diethyl ether (346136-1L, Sigma-Aldrich, St. Louis, MO), vortexed for 1 min, and centrifuged at 3800 rpm for 10 min at 4 °C. This process was repeated twice more, for a total of three diethyl ether washes and the supernatants from each wash were combined in a separate tube and left open in a fume hood. After complete evaporation of supernatant, samples were resuspended in 100 or 175 µL of 1x PBS, transferred to 1.5 mL tubes and stored at -80 °C.

Cortisol analysis was conducted using an enzyme linked immunosorbent assay (ELISA) kit (Salimetrics, Carlsbad, CA, USA) following the manufacturer instructions. Samples were run in duplicate and cortisol concentrations (ng dL^{-1}) were calculated using 4 parameter logistic

regression. Cortisol concentrations were normalized for sample mass to achieve values in ng g^{-1} (ng of cortisol per g of fish tissue).

Metabolite Extraction and Assays

Metabolite (glucose and lactate) determinations followed methods in Bergmeyer (1983) and Fangue et al. (2008). Powdered fish tissue was homogenized in 1 or 0.5 mL of 8% perchloric acid (244252**,** Sigma-Aldrich, St. Louis, MO, USA) in a polytron-type homogenizer over ice for 18 sec. Homogenates were then centrifuged at 10000 RCF for 10 min at 4 °C. Supernatants were removed and immediately neutralized with 54-58 µL of a 3M potassium carbonate solution (347825, Sigma-Aldrich, St. Louis, MO, USA), vortexed, and centrifuged again at 10000 RCF for 10 min at 4 °C. Supernatants were then transferred to 1.5 mL tubes and stored at -80 °C.

For glucose, 20 µL of sample and standard (G8270, Sigma-Aldrich, St. Louis, MO, USA) were added to wells followed by 100 µL of assay buffer (M8266, S5881, A7699, N0632, G8404-2KU ; Sigma-Aldrich, St. Louis, MO). Samples were shaken at 250 rpm and incubated for 5 min at 37 °C before an initial absorbance was read spectrophotometrically at 340 nm. 2 µL of Hexokinase at 2500 U/mL (H4502-2KU, Sigma-Aldrich, St. Louis, MO) was then added to all samples and standards, and plates were shaken at 250 rpm then incubated for 20 min at 37 °C before a second absorbance was taken. The final absorbance (Δ Abs) was calculated as the difference between the first and second absorbance readings. Using the standard curve, glucose (µg mL^{-1}) was calculated from Δ Abs and was normalized for sample mass and expressed in µgg^{-1} (µg of glucose per g of fish tissue).

For lactate, 20 µL of sample and standard (L7022, Sigma-Aldrich, St. Louis, MO) were added to wells followed by 250 µL of assay buffer (N8285, G5418, L3916, Sigma-Aldrich, St. Louis, MO). Samples were then shaken at 250 rpm for 5 min and incubated for 40 min at 37 °C.

Absorbance was then read spectrophotometrically at 340 nm. Using the standard curve, lactate concentration ($\mu g\ mL^{-1}$) was calculated from the absorbance values and was normalized for sample mass and expressed in $\mu g\ g^{-1}$ (μg of lactate per g of fish tissue).

Gill Processing and SEM Imaging

Scanning electron microscopy (SEM) processing was modified from methods described in Wegner et al., (2013) and Kwan et al., (2019). To prepare samples for SEM, samples were gradually dehydrated in a series of ethanol washes (30%, 50%, 70%) at 10 min per wash. At the 70% ethanol mark, delta smelt gills were dissected with the assistance of a microscope, and filaments were trimmed to expose the ionocytes localized along the gill trailing edge. Next, samples were further dehydrated in a series of 10-min ethanol washes (90%, 95%), followed by three consecutive 10-min washes at 100% ethanol. Samples were then immersed in a 50:50 solution of 100% ethanol and hexamethyldisilazane (H1007, Spectrum Chemical, New Brunswick, NJ) for 10 min, followed by three consecutive 10-min washes at 100% hexamethyldisilazane. Samples were dried by evaporation, mounted onto aluminum stubs with double-sided carbon tape, sputter coated with gold palladium, and stored in a desiccator until imaging.

Sample imaging was conducted on two different scanning electron microscopes. We initially imaged using the ThermoFisher Quattro S under low vacuum (10 kV, 40-50 pA) at the UC Davis Advanced Materials Characterization and Testing (AMCaT) Laboratory. This microscope needed repairs during the middle of the analysis period, so imaging continued on the FEI Nova NanoSEM 430 at the UC Davis Center for Nano-MicroManufacturing (CNM2) under vacuum (5 kV, 40-50 pA). All images were captured at 12500x or 25000x magnification. In total, we randomly selected and imaged at least three ionocytes per filament, and at least three

filaments per individual fish. Data from the ≥ 9 ionocytes analyzed for each fish were averaged

for data analysis. Three fish each from enclosures 5-8 in the field and three from the lab

enclosures were analyzed at a subset of time points (0, 1, 27, 48 hours after release). This

resulted in a total of six fish analyzed per density treatment per subset time point from the field

location, and three from the lab.

Microvilli Subtype Identification, SEM Quantification, and Quality Control

In total three types of microvilli were identified, and categorized as *Type I*, *II*, and *III*

(Figure 2). *Type I* microvilli resemble the classic structures observed in prior studies: relatively

short and evenly distributed across the cell surface (Fernandes 2019). *Type II* microvilli were

often multi-pronged and the longest of the three subtypes, and they were usually near the border

of the cell and often formed a ring around the *Type III* microvilli. *Type III* microvilli were

clustered together, often (but not always) within the center of *Type II* microvilli.

SEM quantification methods were modified from the methods described in Skelton et al.,

(2024). The image analysis software FIJI (version 2.1.0; Schindelin et al., 2012) and plugin *Cell*

Counter were used to quantify apical pit and microvilli surface area (SA, μm^2). Apical pit SA

and perimeter were measured by tracing the outer edge of the ionocyte. For every subtype,

microvilli SA was estimated as a cylinder (πrh), which required the number of microvilli using

Cell Counter as well as the measurement of average microvilli height (h) and radius (r)

(estimated from 10 microvilli per subtype, per ionocyte). If less than ten microvilli of a given

subtype were available, then the maximum number of clear qualifications were taken. The total

SA is the sum of the apical pit SA and microvilli SA of all three subtypes. Treatments were

blinded prior to measurements and completed by three different readers for quality control

purposes. Significant differences across readers were determined with visual inspection using

ggplot and with Analysis of Variance (ANOVA) at an α level of 0.05. If detected, readers would re-quantify the images until they agreed. The reported measurements are the averages of the final values recorded by the three independent readers.
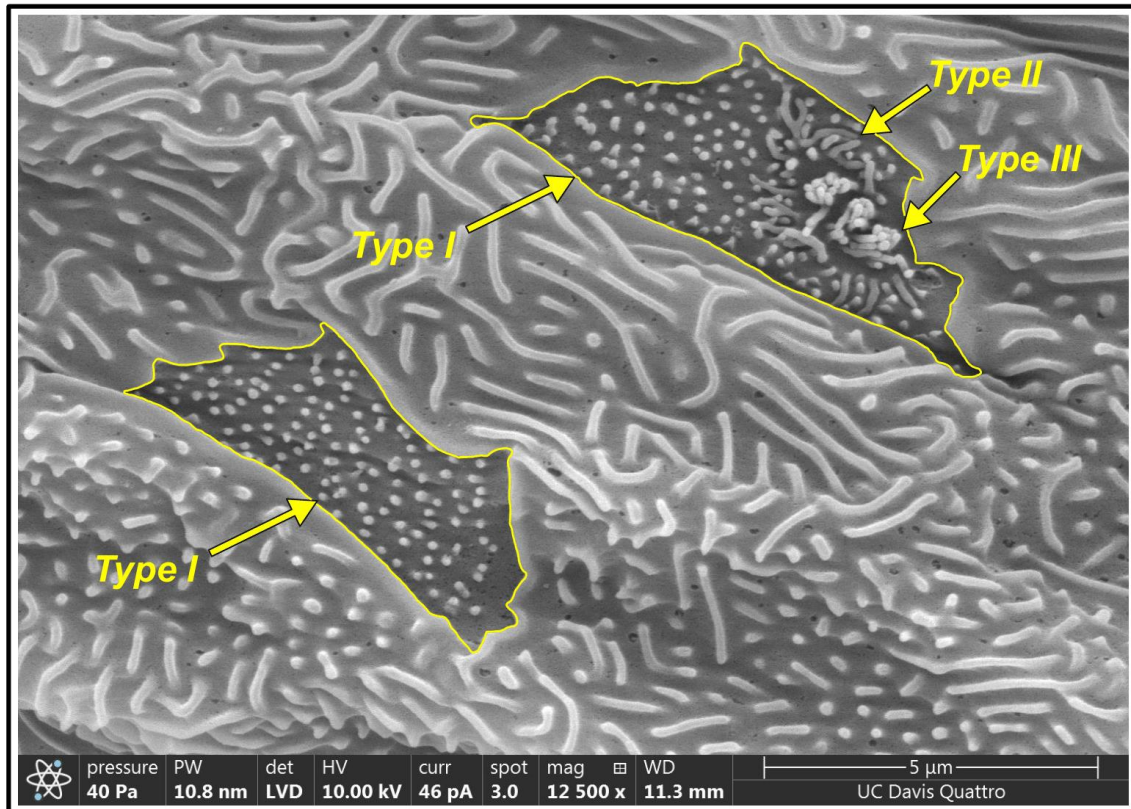
Figure 2: Scanning electron microscopy (SEM) images (25000x) of gill ionocyte apical pit area and the three microvilli types observed and used in analysis. Apical pit area is indicated by the yellow outlines. *Type I* microvilli (A) are short and evenly distributed. *Type II* microvilli (B) are branching structures that spread across the surface of the ionocyte. *Type III* microvilli are elongated and tightly clustered, often found surrounded by *Type I* and *Type II*.

Statistical analysis

Linear mixed effects models (LMMs) were constructed to test for the effect of time point, fish density, and their interaction on temperature, dissolved O_2, pH, alkalinity, and pCO_2 in carboy water. The categorical 'time point' variable distinguishes between the start and end of carboy transport and the 'density' variable represents either the low or high density of fish in carboys. Separate LMMs were constructed with the same design for each water quality metric.

To evaluate differences in cortisol, glucose, and lactate between time points (hours after release to enclosures) and the control group, we constructed a series of LMMs, followed by an ANOVA and custom pairwise contrasts using the *emmeans* package (R version 4.2.3, R Core Team). The LMMs included the response variable of cortisol, glucose, or lactate, regressed against the fixed effects of hours after release to enclosures, fish density, and location, along with the interaction of hours after release and density, and hours after release and location. The categorical 'hours after release' variable represents the discrete time point following release to enclosures at which fish were sub-sampled and included the control. The 'density' variable distinguishes between the high (300 fish per enclosure) and low densities (200 fish per enclosure). The 'location' variable distinguishes between enclosures at the field and lab locations, and the control group. Additionally, a random intercept term was included to account for the repeated sampling of fish from replicate enclosures. ANOVAs on these models for cortisol, glucose and lactate revealed that the fixed effects of density and the interaction of hours after release and density were insignificant for each metric. These effects were removed from the models in order to pool sample sizes across density treatments to improve statistical power between the remaining effects in a final ANOVA. Post–hoc pairwise contrasts were then evaluated for pairwise relationships among hours after release within each site, and contrasts

were also evaluated for pairwise relationships between sites at the same time points. The Bonferroni correction method was applied to resulting p-values to account for the simultaneous testing of multiple comparisons, α remained at 0.05. A compact letter display (CLD) was made to visually interpret pairwise comparisons.

Additionally, we constructed Generalized Additive Mixed Models (GAMMs) to investigate the dynamics of cortisol, glucose, and lactate levels through time, while considering potential variation between the field and lab locations. The GAMMs were formulated using data from hour 0 to hour 27 of the experiment, before the wind event occurred, during which time our experimental design and statistical power were most robust. This was done to better analyze the short-term dynamics of the stress response. Continuous values of cortisol, glucose, or lactate, were regressed on a smooth term for hours after release, allowing for flexible modeling of the non-linear relationship over time. Additionally, we incorporated an interaction term between hours after release and location to assess whether the relationship between cortisol, glucose, or lactate and time varied between locations. A parametric term for location was also included to assess differences between locations. The GAMMs were fitted using the number of splines identified by Akaike's Information Criterion (AIC) to optimize the model fit.

To assess the response of gill ionocyte morphology to shifting water chemistry across densities and locations, we utilized a combination of LMMs followed by ANOVA and post-hoc custom contrasts, and one-way t-tests. LMMs were constructed for total ionocyte surface area (SA, μm^2) of ionocytes, and apical pit SA, and *Type I* microvilli. These models assessed the fixed effects of hours after release, density, and location, along with the interaction of hours after release and density, and hours after release and location, for each response metric. ANOVAs were run on each LMM to assess the relative significance of the effects. Post-hoc contrasts were

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then evaluated for pairwise relationships among hours after release within each site and between density treatments for total ionocyte SA, apical pit SA, and *Type I*. The Bonferroni correction method was applied to resulting p-values to account for the simultaneous testing of multiple comparisons, α remained at 0.05. Contrasts were also evaluated for pairwise relationships between sites at the same time points for the low density treatment. Because the data structure for the Type II and III microvilli SA violated assumptions of homoscedasticity (mean = 0 and sd = 0 at one or more levels), one-way t-tests were constructed between the observed mean of *Type II and III* microvilli SA at each density and time point combination and a hypothesized mean of 0 to represent the control. T-tests were carried out simultaneously for each location and the Bonferroni correction was applied to resulting p-values, α remained at 0.05.

Model assumptions including linearity, normality of residuals, and homoscedasticity were verified visually using diagnostic plots. Data was log-transformed when needed to meet model assumptions. All data are presented as the mean ± standard error of the mean (s.e.m), and α was set to 0.05 for all comparisons. All statistical analyses were performed in R version 4.2.3 (R Core Team).

Results

Carboy Water Chemistry

From the start of carboy transport to the end, dissolved O_2 and pH significantly decreased (t = -2.87, p < 0.001 ; t = -23.12, p < 0.001) and pCO_2 significantly increased (t = 14.12, p < 0.001 ; Table 1). Dissolved O_2 and pH significantly decreased in both low and high density carboys, and the effects were not significantly different between densities (t = 0.70, p = 0.51 ; t = 0.69, p = 0.50). pCO_2 increased in both density treatments and the effect was significantly

greater in the high density carboys (t = 2.34, p = 0.04). Temperature and alkalinity did not significantly change from the start to end of transport (t = -1.65, p = 0.15 ; t = -0.01, p = 0.99) or between densities (t = 0.70, p = 0.51; t = -0.03, p = 0.98).

Factor - Timepoint	Salinity (ppt)	Temperature (°C)	O_2 (mgL^{-1})	pH	Alkalinity (mgL^{-1})	pCO$_2$ (mmHg)
Low - Start	5.00	12.20 (0.10)	19.54 (0.33)	7.92 (0.02)	98.12 (2.69)	194.60 (12.21)
High - Start	5.00	12.10 (0.03)	19.20 (0.19)	7.82 (0.02)	104.53 (0.46)	287.40 (19.62)
Low - End	5.00	12.10 (0.04)	10.69 (0.28) *	7.20 (0.10) *	98.10 (2.25)	1548.00 (88.21) *
High - End	5.00	12.10 (0.10)	9.65 (0.20) *	7.02 (0.02) *	104.50 (4.14)	1958.30 (54.66) *
Field - Release	0.10	10.50	10.96	7.85	68.00	317.40
Lab - Release	0.30	11.30	11.30	8.00	100.00	327.40

Table 1: Carboy water chemistry parameters for both transport densities (Low, High) measured at start of transport (Start), end of transport (End), and environmental conditions in the field (Field) and Lab (Lab) upon release of fish to enclosures (Release). Temperature, O_2, pH, and pCO$_2$ was measured in all 9 carboys and alkalinity was measured in 3 carboys from each fish density treatment. Significant differences ($p < 0.05$) between starting and ending values at each density are indicated by asterisks (*) after end values. Data for carboys is presented as mean (s.e.m). Data for the field and lab environmental conditions were single measures.

Mortality

Total fish mortality (combining mortality observed during sampling and at enclosure removal) was ≤ 3% across all enclosures in the field and lab. For enclosures that were removed before the wind event (following 27 hour time point), we observed 2.25% total mortality in the low density enclosures and 2.67% total mortality in the high density enclosures. For enclosures that were removed at the end of the experiment (following the 72 hour time point) we observed 1.75% total mortality in the low density enclosures and 2.0% total mortality in the high density enclosures. Mortality data is available in Supplementary Table 1.

Cortisol

Cortisol levels varied significantly across hours after release to enclosures (ANOVA; $F = 19.34_{10, 235}$, $p < 0.01$; Figure 3) and there was a significant interaction between location and hours after release (ANOVA; $F = 3.11_{9, 235}$, $p < 0.01$). Pairwise analysis between locations across discrete hours revealed that field and lab cortisol were significantly different only at the 27 hour mark when lab cortisol was higher than the field (pairwise analysis; $t = -3.36_{123}$, $p = 0.01$).

In the field, cortisol increased ~60x from control levels to a peak at 0.5 hours after release (60.70 ng g^{-1} ± 8.13; Figure 4) and these levels were significantly elevated from the control (pairwise analysis; $t = -12.02_{46}$, $p < 0.01$). Cortisol levels significantly decreased from peak levels by 20 hours after release (pairwise analysis; $t = 3.82_{229}$, $p < 0.01$), but remained significantly elevated from the control until 72 hours after release (pairwise analysis; $t = -2.85_{67}$, $p = 0.32$).

At the lab, cortisol increased ~40x from control levels to a peak at 0 hours after release (47.40 ng g^{-1} ± 10.20), and this peak was significantly higher than the control (pairwise analysis; $t = 7.71_{69.3}$, $p < 0.01$; Figure 4). The general trend was for decreasing levels of cortisol in the lab,

but from the 6 to 20 hour mark cortisol nearly doubled (pairwise analysis; $t = 2.09_{228}$, $p = 1.00$). Cortisol in the lab was both significantly decreased from peak levels and no longer significantly elevated from control levels by 48 hours after release (pairwise analysis; $t = 4.10_{228}$, $p < 0.01$; $t = 2.38_{88}$, $p = 1.00$).

Cortisol recovery over time in delta smelt in the field and lab followed a nonlinear relationship (Figure 4). The smoothed term for hours after release, and the interaction between location and hours after release were both significant predictors of cortisol (GAMM; $F = 42.72$, $p < 0.01$; $F = 5.01$, $p < 0.01$) and the parametric coefficient of location was not significant (GAMM; $t = -0.02$, $p = 0.98$).

Glucose

Glucose levels in delta smelt varied significantly across hours after release into enclosures (ANOVA; $F = 3.92_{10,59}$, $p < 0.01$; Figure 3). However, there was no significant interaction between hours after release and location (ANOVA; $F = 1.30_{9, 232}$, $p = 0.24$).

In the field, glucose peaked to levels double the control by 0.5 hours after release (119.20 $\mu gg^{-1} \pm 10.17$), but pairwise analysis showed this was not significantly higher than the control (pairwise analysis; 63.20 μg $g^{-1} \pm 6.58$, $t = 3.93_{17}$, $p = 0.06$). Glucose at 1 hour was very similar to the peak at 0.5 hours (117.26 $\mu gg^{-1} \pm 7.25$) and yet pairwise analysis showed it was significantly higher than the control (pairwise analysis; $t = 4.07_{17}$, $p = 0.04$). Glucose was significantly elevated from the control until 3 hours after release (pairwise analysis; $t = 3.52_{17}$, $p = 0.14$), and was significantly decreased from peak levels by 27 hours (pairwise analysis; $t = 5.09_{232}$, $p < 0.01$).

At the lab location, glucose peaked to levels ~1.5x the control by 0 hours after release (101 $\mu gg^{-1} \pm 12.50$), but this was not significantly higher than the control (pairwise analysis; $t =$

2.17_{24}, p = 1.00). No pairwise contrasts between hours after release in the lab were significantly different for glucose. Similar to cortisol, from the 6 to 20 hour mark in the lab glucose showed a non-significant increase (pairwise analysis; $t = 2.12_{232}$, p = 1.00).

The recovery of glucose from peak levels followed a nonlinear relationship to hours after release (Figure 4). The smoothed term for hours after release was a significant predictor of glucose (GAMM; F = 8.54, p < 0.01), but the interaction between the field and lab site was not (GAMM; F = 3.18, p = 0.15). The parametric coefficient of location was a significant predictor of glucose (GAMM; t = 155.50, p < 0.01).

Lactate

Lactate levels in delta smelt varied across hours after release into enclosures (ANOVA; F = $2.80_{10, 90}$, p < 0.01; Figure 3), and there was no interaction between hours after release and location (ANOVA; F = $0.77_{10, 241}$, p = 0.65). In the field, lactate peaked to levels ~1.4x the control by 0.5 hours after release (565.14 $\mu gg^{-1} \pm 36.88$) but this increase was not significantly greater than the control (pairwise analysis; $t = -1.72_{10}$, p = 1.00). Lactate was not significantly different from the control at any other hour. Peak lactate was significantly decreased in field enclosures by the 6 hour mark (pairwise analysis; $t = 6.14_{241}$, p < 0.01), when lactate had fallen to levels 1.6x below the control (pairwise analysis; 355.28 $\mu gg^{-1} \pm 36.71$). At the lab, lactate levels peaked at 0 hours after release (471.10 $\mu gg^{-1} \pm 53.91$) and this was not significantly different from the control (pairwise analysis; $t = -0.57_{12}$, p = 1.00). No other pairwise comparisons between hours in the lab were found to be significant.

The recovery of lactate from peak levels followed a nonlinear relationship with hours after release in the field (Figure 4). The smoothed term for hours after release was a significant predictor of lactate (GAMM; F = 8.01, p < 0.01), and the interaction between the field and lab

site was not a significant predictor (GAMM; $F = 2.21$, $p = 0.38$). The parametric coefficient of location was significant for lactate (GAMM; $t = -2.08$, $p < 0.01$).
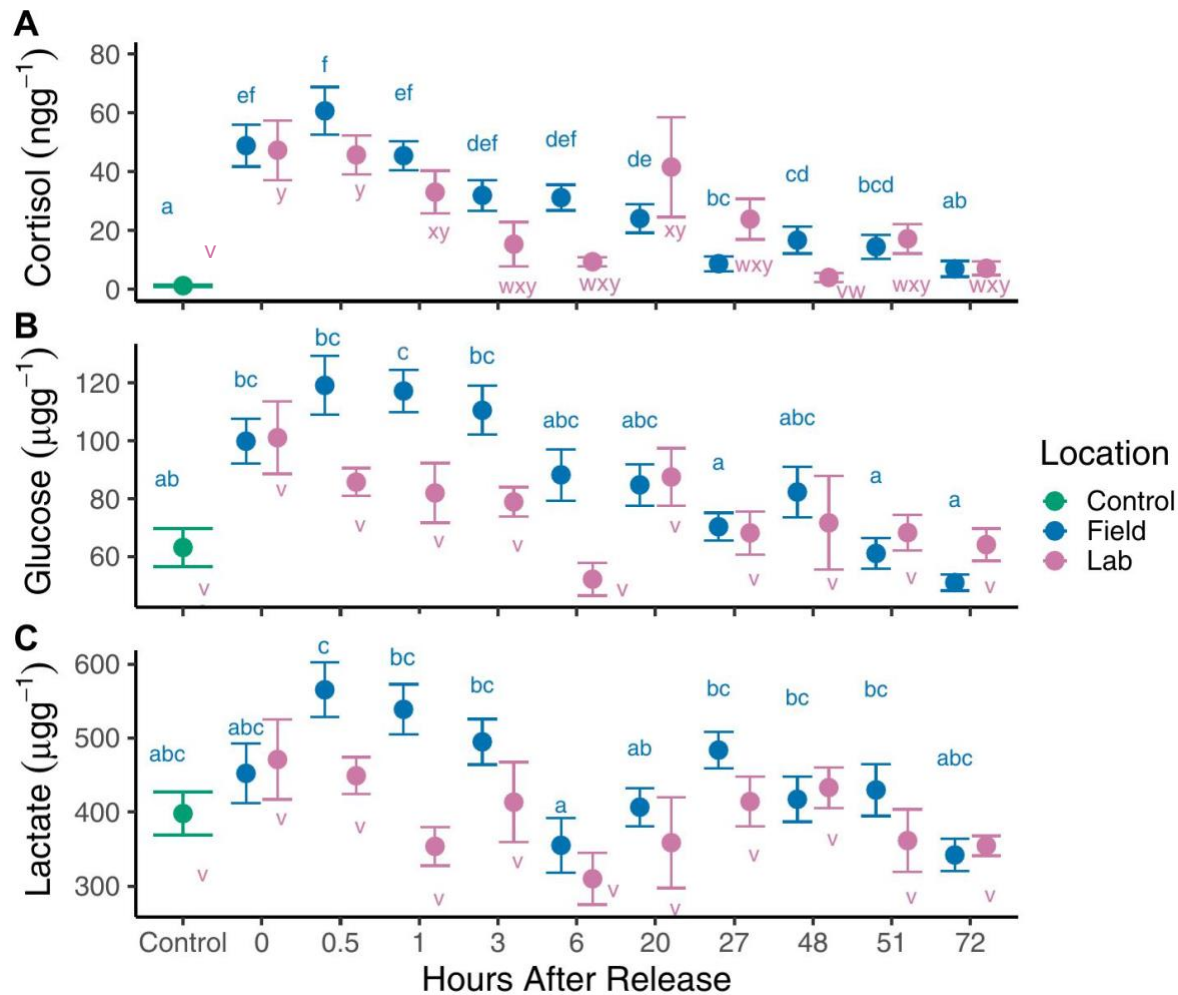
Figure 3: *Average whole-body cortisol (ng g^{-1}; A), glucose (µg g^{-1}; B), and lactate (µg g^{-1}; C) across the experimental period compared to control levels.* Blue denotes data from the field and pink represents data from the lab. Blue letters (a,b,c,d,e,f) represent pairwise contrasts within the field location, pink letters (v,w,x,y,z) indicate pairwise comparisons within the lab location. Differences in letters represent results from the planned contrast testing. Asterisks (*) indicate significant differences between locations at individual time points using pairwise contrasts. Points, bars, and letters for the field and lab are jittered for ease of viewing. Data is represented as mean ± s.e.m.
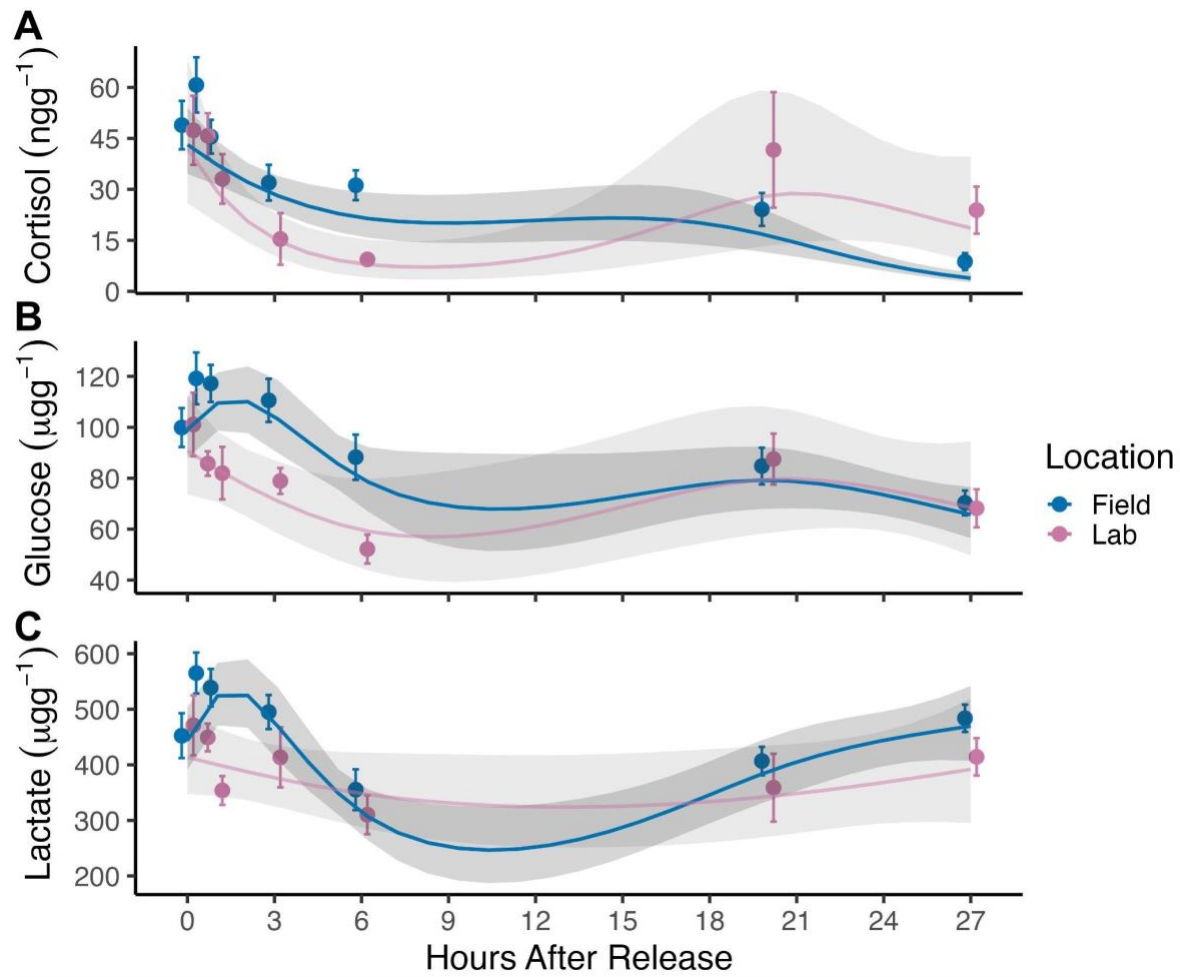
Figure 4: Generalized additive mixed modeling (GAMM) for whole-body cortisol (ng g⁻¹; A), glucose (μg g⁻¹; B), and lactate (μg g⁻¹; C) over the first 27 hours of the experiment. Blue corresponds to data from the field location, while pink represents data from the lab location. Points and bars represent means ± s.e.m. Gray ribbons represent 95% confidence intervals surrounding the GAMM model output.

Density - Location	Total Mortality During Sampling	Total Mortality at Enclosure Removal	Average Total Mortality Per Enclosure (%)
Low - Field	12	4	2.00
High - Field	14	17	2.33
Low - Lab	0	0	0

Supplemental Table 1: Total observed mortality of delta smelt in cages during sampling, upon removal of cages, and the average percentage of morality per cage density - location combination.

Metric	Terms	e.d.f	F-value	P-value
Cortisol	f(H) for Field	2.90	42.72	< 0.01
	Deviance from f(H) for Lab	2.87	5.01	< 0.01
Glucose	f(H) for Field	4.26	8.54	< 0.01
	Deviance from f(H) for Lab	2.78	3.18	0.15
Lactate	f(H) for Field	4.57	8.01	< 0.01
	Deviance from f(H) for Lab	1.78	2.21	0.38

e.d.f., effective degrees of freedom.

Supplemental Table 2: Comparisons of smoothed terms of generalized additive mixed models of cortisol, glucose, and lactate as a function of hours since release, f(H) referenced to the field location.

Gill Ionocyte Morphology

Total gill ionocyte SA varied significantly across hours after fish were released into enclosures (ANOVA; $F = 5.82_{4,46}$, $p < 0.01$; Figure 5) and was significantly affected by the interaction between hours after release and location (ANOVA; $F = 8.23_{3,46}$, $p < 0.01$) and the interaction between hours after release and density (ANOVA; $F = 3.67_{3,46}$, $p = 0.02$). In the high density enclosures in the field, total gill ionocyte SA was 2.5x higher than control levels by 1 hour after release (pairwise analysis; $t = 3.90_{19}$, $p = 0.03$), and peaked to levels triple the control by 48 hours after release (pairwise analysis; $t = 4.43_{19}$, $p = 0.01$; Figure 5). In the low density enclosures in the field, peak total gill ionocyte SA ($13.72 \ \mu m^2 \pm 2.06$) at 1 hour after release was not significantly elevated from the control (pairwise analysis; $t = 3.09_{19}$, $p = 0.22$). In the lab, total gill ionocyte SA peaked to levels 4x the control by 27 hours after release (pairwise analysis; $t = 4.96_{19}$, $p < 0.01$). When comparing across the field and lab locations, total gill ionocyte SA was twice as high in the field at 1 hour after release (pairwise analysis; $t = 2.84_{19}$, $p = 0.04$), and was ~1.5x higher in the lab at 27 hours after release (pairwise analysis; $t = -2.91_{21}$, $p = 0.03$).

The components that make up total gill ionocyte SA include the SA of the apical pit as well as *Type I*, *Type II* and *Type III* microvilli. ANOVA analysis revealed that apical pit, and *Type I* microvilli SA varied significantly across hours after release to enclosures (ANOVA; $F = 5.37_{4,46}$, $p < 0.01$; $F = 5.82_{4,46}$, $p < 0.01$; Figure 6). For apical pit SA, there was a significant interaction between hours after release and density and between hours after release and location (ANOVA; $F = 3.09_{3,46}$, $p = 0.04$; $F = 3.80_{3,46}$, $p = 0.02$). In the field, apical pit SA was significantly greater than control levels by 1 hour after release in high density enclosures (ANOVA; $t = 4.25_{19}$, $p = 0.02$), and by 0 hours after release in the low density enclosures (ANOVA; $t = 3.87_{19}$, $p = 0.04$; Figure 6A). In the lab, apical pit area was significantly elevated

from the control only at the 27 hours time point (ANOVA; $t = 4.12_{19}$, $p < 0.01$; Figure 6B).

Apical pit SA did not significantly differ between the field and lab locations at any hours after release.

For *Type I* microvilli SA, the interaction between hours after release and location was significant (ANOVA; $F = 8.23_{3,46}$, $p < 0.01$), but the interaction between hours after release and density was not significant. In the field, *Type I* microvilli SA did not significantly differ from the control despite values 2x greater than the control in high density enclosures (Figure 6C). In the lab, *Type I* microvilli SA was significantly elevated from the control by 27 hours after release due to a peak 4x higher than the control (pairwise analysis; $t = 3.93_{19}$, $p = 0.01$; Figure 6D). Between field and lab samples, *Type I* microvilli SA was twice as high in the field than the lab at 1 hour after release (pairwise analysis; $t = 3.81_{19}$, $p < 0.01$), and was half as high in the field than lab at 27 hours after release (pairwise analysis; $t = -3.12_{21}$, $p = 0.02$).

No *Type II* microvilli were observed in the control samples. However, average *Type II* microvilli SA gradually increased from ~1 to ~5 μm^2 across the experimental period. In low density enclosures in the field, *Type II* microvilli SA was not significantly elevated from control levels at any hour after release (Figure 6F). In high density enclosures in the field, *Type II* microvilli SA was significantly elevated from the control by 48 hours after release (one-sample t-test; $\mu_0 = 0$; $t = 4.07_5$, $p = 0.03$; Figure 6E). In the lab, *Type II* microvilli were significantly elevated from the control at 27 hours after release (one-sample t-test; $\mu_0 = 0$; $t = 7.20_{12}$, $p = 0.04$; Figure 6F).

No *Type III* microvilli were observed in the control samples, or at 0 hours after release. Average *Type III* microvilli SA was ~2.5 μm^2 at 1 hour after release to enclosures and decreased gradually to ~1 μm^2 by 72 hours after release. In low density enclosures in the field, *Type III*

microvilli were significantly elevated from control levels at 1 hour after release (one-sample t-test ; $\mu_0 = 0$; t=4.2$_5$, p < 0.01; Figure 5G), high density enclosures were never significantly elevated. In the lab, *Type III* microvilli SA were never significantly elevated from the control (Figure 6H).
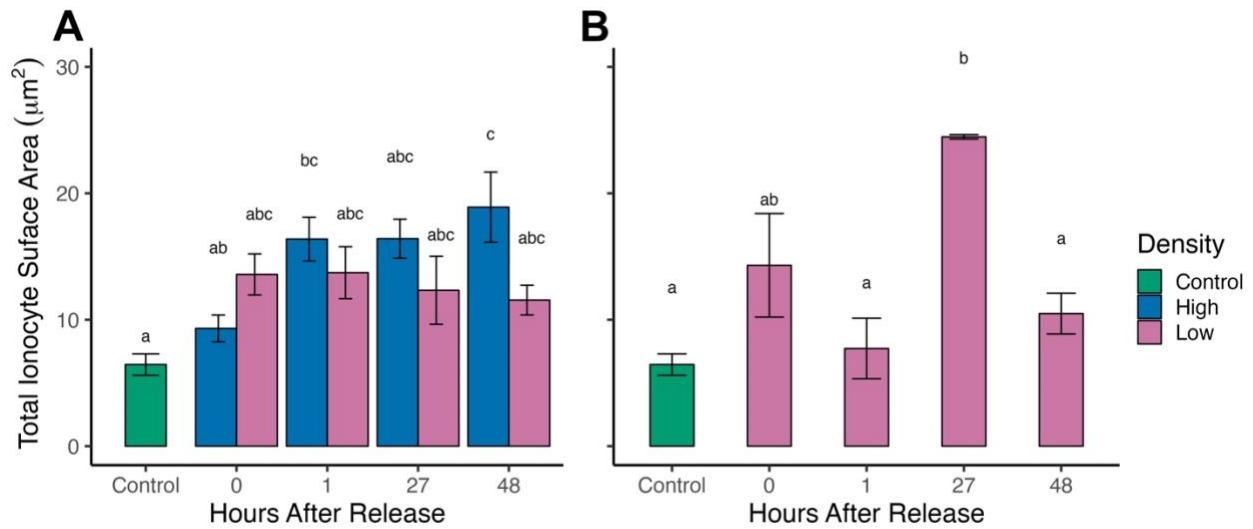
Figure 5: Total gill ionocyte surface area (μm^2) across hours after release, at the field site (A) and lab site (B). Blue represents data from high density enclosures and pink represents data from low density enclosures. Green represents the control group and is identical for both field and lab. Letters represent pairwise comparisons between time points and density treatments. Data is represented as mean ± s.e.m.
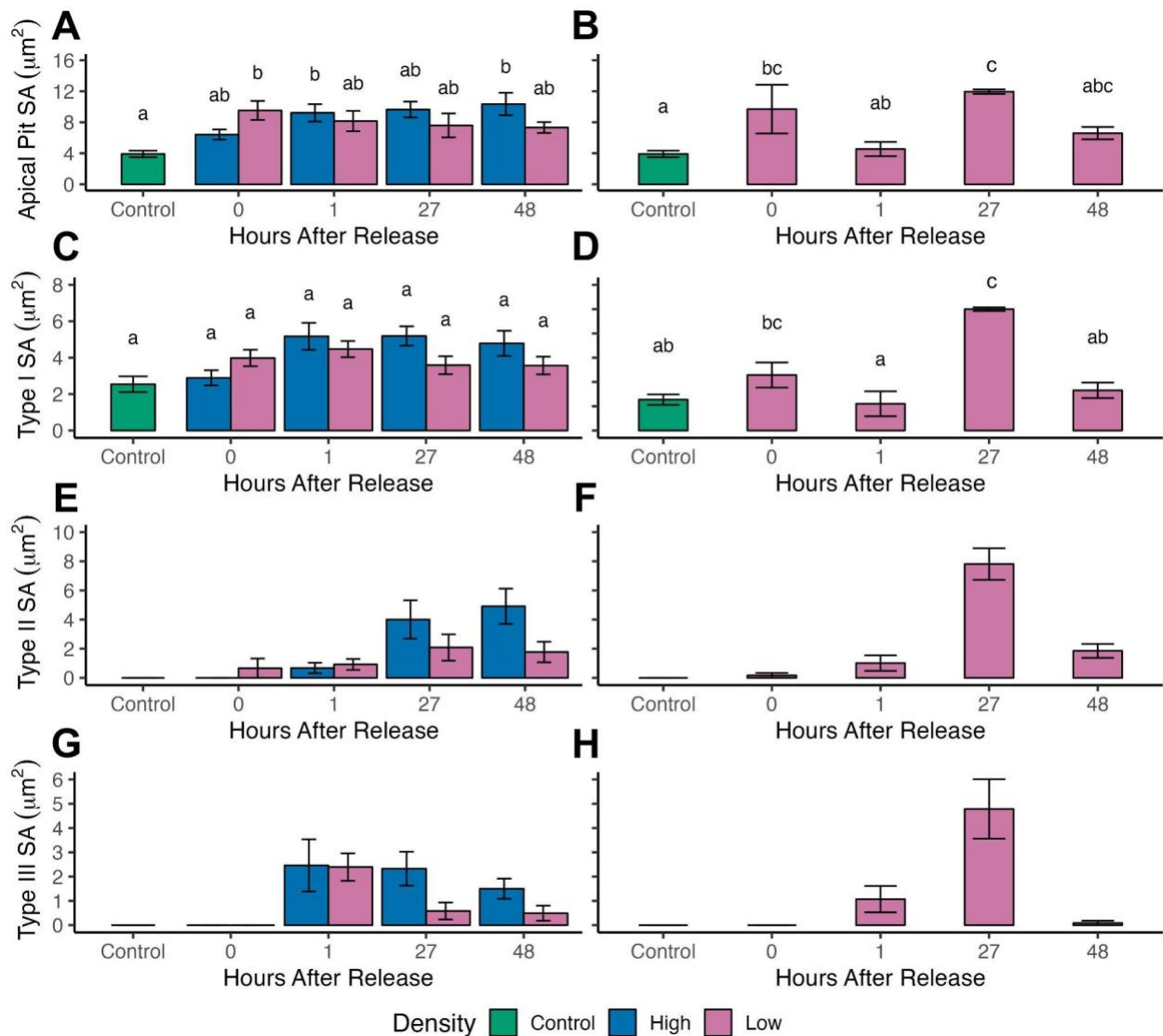
Figure 6: Surface area (SA; µm²) of apical pit, *Type I, II,* and *III* microvilli across hours after release at the field site (A, C, E, G) and lab site (B, D, F, H). Blue represents data from high density enclosures and pink represents data from low density enclosures. Green represents the control group and is identical for both field and lab. Letters represent pairwise comparisons between time points and density treatments for apical pit and *Type I* microvilli (ANOVA), asterisks indicate significant differences from zero for *Type II* and *III* microvilli (one-way t-tests, with multiple comparisons adjustment). Data is represented as mean ± s.e.m.

Discussion

Delta smelt experience a multitude of physiological stressors during transport and release into the wild. Responses to these stressors could determine the short and long term performance of released delta smelt by limiting energy for essential functions. Swimming ability (Strange and Cech 1992, Lankford et al., 2002, Milligan 2003, Carbonara et al., 2010), predator avoidance (Jarvi 1990, Olla and Davis 1989, Olla et al., 1995, Danylchuk et al., 2007), feeding (Gregory and Wood 1999, Carr 2002, Bernier 2006, Conde-Sieira et al., 2018), and immune function (Tort 2011, Nardocci et al., 2014) are all examples of nominal function inhibited during acute or chronic stress. Stress also inhibits reproductive functions and output (Mommsen et al., 1999, Schreck et al., 2001, Schrek 2010), such as reduced fecundity and quality of eggs (Campbell et al., 1992, 1994, Schreck et al., 2001, Milla et al., 2009, Mileva et al., 2011, McConnachie et al., 2012), which can reduce recruitment of a population. These effects could be particularly potent for delta smelt, a semelparous species with a one year life cycle that is released shortly before their reproductive period. Therefore, quantifying stress during the release process is essential for determining the efficacy of delta smelt supplementation.

Our study quantified a significant elevation of cortisol, glucose, and to a lesser degree lactate, following release to enclosures indicating that a substantial stress response was elicited from supplementation methods. Our analysis of gill ionocyte morphology revealed a significant increase in total ionocyte SA in response to IOA-B disturbance during transport and release to the SSJD. Ionocyte SA was positively correlated with cortisol and the presence of novel microvilli types and their differential use through time indicate that microvilli types serve different functions. To the best of our knowledge, this study is the first to report multiple types of microvilli on the apical surface of individual gill ionocytes. Our results indicate that recovery of

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stress metrics, including cortisol, glucose and lactate, is achieved after ~27 hours when using the soft release methodology, and that delta smelt continue to utilize IOA-B regulatory mechanisms to acclimatize to field conditions.

Cortisol

The timing and magnitude of cortisol circulation in response to general stressors varies widely across fish species and life histories (Pankhurst 2011). Significant cortisol elevation has been observed as early as 2.5 min after a stressor in striped bass *Morone saxatilis* (Tomasso et al. 1996) and as late as 2.5 hours in sea raven *Hemitripterus americanus* (Vijayan and Moon 1997). The magnitude of plasma and whole-body cortisol increase following stressors varies 100x (~3-300 ngmL^{-1}) across teleost and elasmobranch fishes and the typical recovery time to baseline levels occurs anywhere from 2-48 hours (reviewed in Barton 2002 and Pankhurst 2011).

In the only other study on the whole-body delta smelt stress response, cortisol peaked to levels ~20x the control after 15-30 min following acute handling (Pasparakis et al. 2016). Comparatively, the ~60x increase in whole-body cortisol observed here by 30 minutes after release likely reflects the accumulation of stressors faced by delta smelt during full scale supplementation versus a single handling event. Cortisol levels observed here are relatively high compared to most studies using 2 hour transport periods (Sampaio and Freire 2016). For example, in a study on four trout species following acute handling and 2 hour transport, peak cortisol was reached 3 hours after release and the peak from control levels was 30-40x higher than the control (Barton 2002). The relatively high cortisol observed here is likely indicative of the known sensitivity of delta smelt to a variety of stressors, and the high mortalities often attributed to handling (Swanson et al. 1996; Connon et al. 2009, 2011a, 2011b). However, we

did not observe high mortality following release, suggesting that the high levels of cortisol observed are within the allostatic limit of delta smelt.

Cortisol in the field showed a greater peak and slower initial recovery than in the lab likely reflecting differing environmental conditions. Any number of extraneous environmental stressors present in the field, such as predator cue, changes in turbidity, or effects of enclosures could have increased cortisol in the field and hampered recovery (Hasenbein et al., 2013, Pasparakis et al., 2016). Additionally, environmental salinity in the lab was higher (0.3 ppt) than in the field (0.1 ppt). High salinity has been shown to reduce cortisol expression and improve survival for transport of delta smelt and other species (Swanson et al. 1996, Cech et al. 1996, Portz et al., 2006). This effect is likely due to the role of cortisol in regulating IOA-B regulation during stress (more in the "*Gill Ionocyte Morphology*" section). Therefore, the higher environmental salinity present in the lab, although modest, could have contributed to more rapid recovery of cortisol than in the field.

For fish in the lab, cortisol rapidly increased at the 20 hour mark, then decreased back to baseline levels by 48 hours. Shortly before sampling at 20 hours after release, a septic tank near the outdoor recirculating tank at the lab location was being pumped by a large truck (L. Ellison, personal communication, February 16, 2024). The noise and vibrations associated with this pumping could have led to an increase in cortisol at the 20 hour time point. No other stressors were known to occur besides this. This sudden increase in cortisol was followed by rapid recovery and no increase in mortality.

In contrast to past studies (Ruane et al., 2002, Liu et al. 2016), we did not see an effect of carboy and enclosures density on cortisol circulation. Given the volume of carboys (75 L) and enclosure size (height: 1.2 m, diameter: 1 m), the difference between density treatments (200 vs

300 fish) may not have been sufficient to cause a difference in cortisol expression. It is also possible that our transport period (~2 hours) was not long enough to cause a difference in the accumulation of ammonia between high (4 fish per liter) and low (~2.6 fish per liter) density carboys that would be expected to elicit a cortisol response (Williams et al. 2017). Our findings support the use of higher density transport containers that would reduce costs and logistical challenges required for transporting delta smelt, thereby increasing the efficiency of the supplementation effort.

Overall, our cortisol results indicate a significant activation of the HPI axis in delta smelt in response to the suite of stressors during the supplementation process. Recovery of cortisol levels by 20 hours, and further by 72 hours after release, and minimal mortality indicate that this stress response is within the allostatic capacity of delta smelt and that physiological homeostasis can be re-established using the soft release methodology. Our data indicates that the release of delta smelt without allowing time for stress recovery, as is done in the hard release method, may result in poor performance of released fishes due to high levels of cortisol and associated energetic costs during release to a novel environment. In contrast, the use of *in-situ* enclosures for delta smelt alleviates this effect by allowing time for stress recovery and could result in higher post-release recapture rates and site fidelity as has been shown in other studies on *in-situ* acclimation (Jonssonn et al., 1999, Brennan et al., 2006, Kuwada et al., 2004). Our results also suggest that lower salinity at the field release site may hamper the recovery of stress. Therefore, our data suggest that soft release supplementation should allow for at least 20 hours of enclosure acclimation. Future research should seek to elucidate sub-lethal impacts of increased cortisol on performance and fitness after release from enclosures.

Glucose & Lactate

An increase in the circulation of glucose and lactate has been shown to follow elevated cortisol levels during transport stress in many but not all fish species (Barton 2000, Acerte et al., 2004, Pasparakis et al., 2016, Jiang et al., 2017). The degree of increase likely depends on the length and severity of the stressor and the degree of energy demand and anaerobic metabolism (Wood et al. 1983, Barton and Iwama 1991, Barton 2002, Polakof et al. 2012, Sampaio and Friere 2016). Our data indicate that a rapid mobilization of glucose was needed to fuel rapid metabolism in delta smelt across the supplementation process, and that O_2 demand exceeded supply in some tissues leading to anaerobic metabolism and the accumulation of lactate. In Pasparakis et al. (2016), neither glucose nor lactate in delta smelt showed a significant response to acute handling over the 60 min observation period. The greater increase in glucose and lactate observed here indicates a more substantial stress response to the multiple stressors of soft release versus a single handling event. In eurasian perch (*Perca fluviatilis*) and various trout species subjected to handling and transport for 2-4 hours, researchers observed a 2x elevation in glucose but no increase in lactate (Barton 2002, Acerete 2004). The more pronounced increase in lactate observed here compared to these studies indicates a greater degree of anaerobic metabolism characteristic of intense stress or exercise (Milligan and Girard 1993). This underscores the magnitude of metabolic demand delta smelt experience during the transport and release process. However, our data suggests that a return to nominal levels of energy demand occurred by ~24 hours after release when glucose and lactate levels had fallen substantially.

In conjunction with cortisol, glucose and lactate significantly increased at the 20 hour mark in the lab before decreasing back to baseline levels. This increase in glucose and lactate could have been a direct response of increased cortisol following the septic tank pumping, or

another unknown stressor. Both glucose and lactate recovered back to baseline levels following this acute elevation.

An increase in glucose and lactate commonly follows cortisol in response to high transport and holding densities (Sangiao-Alvarellos et al., 2005, Hong et al. 2019). Given that we did not see a significant response of cortisol to increased density, it is not surprising that glucose and lactate also did not increase. Therefore, we have no evidence that the heightened densities used here elicited any substantial energy mobilization in delta smelt.

Our glucose and lactate results indicate that delta smelt expended their energy stores during the supplementation process. In trout, latent mortality up to 40% following stress was linked to high levels of glucose metabolism and lactate accumulation (Wood et al. 1983). Despite a significant response of glucose, and more moderate lactate increase, we observed less than 3% latent mortality in all enclosures and a rapid recovery of both glucose and lactate. This indicates that the energetic cost of the secondary stress response is not beyond the allostatic capacity of delta smelt, and delta smelt are able to reduce their energy use and reestablish metabolic homeostasis during soft release (Schreck 2010, Van Ginneken et al., 2008). Moreover, the positive growth of delta smelt in enclosures observed in Baerwald et al. (2023) indicates that the rapid replenishment of depleted energy stores is possible after stress recovery.

Similar to cortisol, our metabolite data indicates that the use of the soft release methodology, opposed to hard release, allows for an important period of stress recovery and a return to energetic equilibrium after at least 20 hours of acclimation. This acclimation period may improve performance of delta smelt in the wild by limiting extraneous energetic costs associated with the stress response.

Gill Ionocyte Morphology

Gill remodeling includes the alteration of ionocyte apical morphology to accelerate ion exchange to restore homeostasis (Cameron and Iwama 1987, Cameron 1989, Goss et al., 1992) and has been documented in response to disturbances including harmful algal bloom (Skelton et al., 2024), hypo- and hyper salinity (Varsamos et al., 2002), and dramatic changes in acid-base conditions (Hyde and Perry 1989, Goss et al., 1992a, Perry and Goss 1994, Furukawa et al., 2011, Dymowska et al., 2012). Similar to cortisol, glucose and lactate responses, the general pattern for total ionocyte SA response includes an initial increase that dampens over time. However, we quantified that total ionocyte SA remained relatively higher than control levels throughout the experimental period. This indicates that delta smelt required both short-term IOA-B regulation following transport and long term acclimatization to the dilute salinity levels in the field.

During the initial handling and carboy transport, delta smelt likely experienced both metabolic acidosis and respiratory acidosis leading to blood pH decrease and CO_2 accumulation. Freshwater fish responses to acidosis are well documented (Claiborne and Heisler 1986, Hyde and Perry 1989, Goss et al., 1992a, Hwang 2009, Furkawa et al., 2011): gill ionocytes exchange environmental Na^+ for plasma H^+ to normalize blood pH levels, leading to an accumulation of plasma HCO_3^-. The rate of recovery would not only be expedited by increased ionocyte apical SA and *Type I* microvilli, but also by the greater available counterions (e.g. NaCl) due to salt addition (5.0 ppt; Table 1). We observed a significant increase in apical area and *Type I* microvilli directly following release (0 hours after release) consistent with known mechanisms of acidosis recovery. The lack of *Type II* and *Type III* microvilli in the control and upon release (0 hours after release), and their differential expression over time, indicate that these structures may serve a different purpose than apical area and *Type I* microvilli. Past studies have consistently

reported a single type of microvilli within gill ionocytes (Franklin 1990, Brown 1991, Goss et al., 1994, Varsamos et al., 2002, Fridman et al., 2011, Kwan et al., 2019, Skelton et al., 2024), consequently our discovery of three different microvilli types within a single gill ionocyte was both exciting and entirely unexpected. Notably, Varsamos *et al.* (2002) did show (but did not report or discuss) a second type of microvilli within the same ionocyte in freshwater adapted (0.2 ppt) European seabass (*Dicentrarchus labrax*). Regardless, this study is the first to show and report three types of microvilli within the same gill ionocyte.

Upon release to enclosures, delta smelt were returned to normal pCO_2/pH conditions artificially inducing respiratory alkalosis (Lonthair et al., 2020) and the likely need for fish to offload accumulated plasma HCO_3^-. This IOA-B disturbance is an incredibly rare scenario that is unlikely to be experienced throughout most of the delta smelt's life at the hatchery. This may explain why the two new types of microvilli were not observed in the pre-transport control or directly following release to enclosures (0 hours after release) but were in much greater abundance by 1 hour of enclosure acclimation when IOA-B regulation was likely occurring. Moreover, fish released to the field were exposed to 50x less salinity than they experienced during transport (Carboy: 5.0 ppt, Field: 0.1 ppt). As such, the significantly less counterions in the field likely slowed IOA-B regulation. In contrast, fish released into the lab were exposed to 16.7x less salinity (Carboy: 5.0 ppt; Lab: 0.3 ppt; Table 1). This could explain why fish in the lab showed a generally lower increase in total ionocyte SA, *Type II* and *Type III* microvilli. Altogether, this underscores the importance of considering the salinity levels of field release locations based upon organismal response and rearing conditions.

Although additional experiments will be needed to confirm the role of *Type II* and *Type III* microvilli during the enclosure acclimation period, their differential pattern hints at their roles

in IOA-B regulation. In general, the *Type II* microvilli slowly increased over time after release into field enclosures. This timeline corresponds to the increasing demand to upregulate ion-absorption and maintain internal osmolarity in a low salinity environment. In contrast, the use of *Type III* microvilli peaked 1 hour after release to field enclosures but dampened over time. This timeline would match the need for plasma HCO_3^- removal and acid-base recovery following transport. Finally, the magnitude of the *Type II* and *Type III* microvilli response also matches the salinity levels between lab and field conditions: with the exception of the 27 hour time point where fish were disrupted by the septic truck, *Type II* and *Type III* microvilli response of fish in the lab were generally milder than those in the field.

Cortisol is not only a stress response hormone, but has also been linked to ionocyte responses (Perry et al., 1992, Goss et al., 1994, Evans et al., 2005, Kumai et al., 2012). Cortisol has been correlated to increased Na^+ reabsorption, H^+ excretion, and HCO_3^- accumulation (Kumai et al. 2012, Kwong et al. 2014), as well as Cl^- absorption and HCO_3^- expulsion (Laurent and Perry 1990, Wood et al., 1999). Cortisol is also known to increase surface area and number of gill ionocytes to facilitate ion transport (Laurent and Perry 1990, Goss et al. 1994, 2001, Brannen and Gilmour 2018). Our results indicate that peak cortisol at 0.5 hours after release to the field enclosures could have been partially in response to the need for IOA-B regulation shortly after transport, and corresponds with the significant increase in ionocyte surface area 1 hour after release to enclosures. Moreover, upon the brief increase in cortisol at the 27 hours of enclosure acclimation in the lab, we observed a similar sudden increase in ionocyte surface area.

Together, our gill ionocyte morphology results indicated delta smelt mounted a considerable IOA-B regulatory response to shifting water chemistry across transport and release into the SSJD. Recommendations for future supplementation include utilizing transport

containers that can off-gas CO_2 to limit acidosis during transport and opting for field release locations with higher salinities to provide sufficient counterions to facilitate IOA-B regulation after release.

Conclusion

Our study reveals that delta smelt utilize multiple stress response mechanisms to counteract a suite of physiological stressors and IOA-B disturbances during soft release. We show that the use of acclimation enclosures results in significant stress recovery after 20 hours, and that continued IOA-B regulation is used to acclimatize to field conditions. Together, these data indicate that soft release supplementation provides an effective method for ensuring delta smelt are released into the wild after they have restored physiological equilibrium. Soft release could therefore improve both the efficacy and efficiency of delta smelt supplementation by improving performance and survival in acclimated delta smelt. Future studies should evaluate movement, mortality, and performance of fish used in both hard and soft release to provide a more robust comparison of each method. Identifying the effective method for the release of artificially cultured delta smelt may significantly benefit the conservation of these critically endangered fish.

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