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Evaluating stress recovery and gill morphology during experimental supplementation of an endangered fish species

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Abstract

The efficacy of population supplementation (i.e., stocking) may be limited by physiological stress incurred upon transported and released fishes. In an experimental supplementation program for the critically endangered delta smelt (Hypomesus transpacificus), acclimatization enclosures have been developed to facilitate stress recovery. However, the magnitude and duration of the stress response and the optimal time of release from enclosures remain unknown. To address these knowledge gaps, we quantified whole-body cortisol, glucose, lactate, and gill ionocyte morphology at 10 time points across 72 h following transport to enclosures at high and low densities. Cortisol and glucose peaked within 1 h following transport into enclosures then declined significantly by 27 h. Lactate was moderately affected, and transport density had no effect on cortisol, glucose, or lactate. Gill ionocyte surface area significantly increased in response to shifts in water chemistry throughout transport, and we observed three distinct types of microvilli on the surface of gill ionocytes, including two never previously reported. This work highlights the importance of ensuring stress recovery during the supplementation of delta smelt and other species.

Key words: cortisol, delta smelt, estuary, stocking, reintroduction

Introduction

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, 2013; 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 1998; Seddon et al. 2005; Seddon et al. 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 result in an increase of circulating stress hormones (i.e., glucocorticoids such as cortisol and corticosterone) and a dysregulation of energy balance (Barton and Iwama 1991; Barton 2000; Hartup et al. 2005; Dickens et al. 2010; Jenni et al. 2015; Batson et al. 2017). The stress response is an adaptive trait designed to help animals respond to nominal stressors in their environment (Wendelaar Bonga 1997). However, the stress response can negatively impact behavior, physiological performance, and fitness in the wild if stressors are particularly intense, chronic, or occur at a critical life history stage (Wendelaar Bonga 1997; Teixeira et al. 2007; Dickens et al. 2010; Schreck 2010; Sokolova 2021). Reproduction, feeding, predator avoidance, immune function, and energy balance are examples of adaptive traits inhibited by stressors associated with supplementation (Teixeira et al. 2007; Dickens et al. 2010; Schreck 2010; Sokolova 2021). 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 terrestrial vertebrate and aquatic mammal supplementation, only 9% evaluated physiological metrics, such as the stress response (Tarszisz et al. 2014).

The success of fish supplementation has been mixed (Lamothe et al. 2019). Lake sturgeon (*Acipenser fulvescens*)

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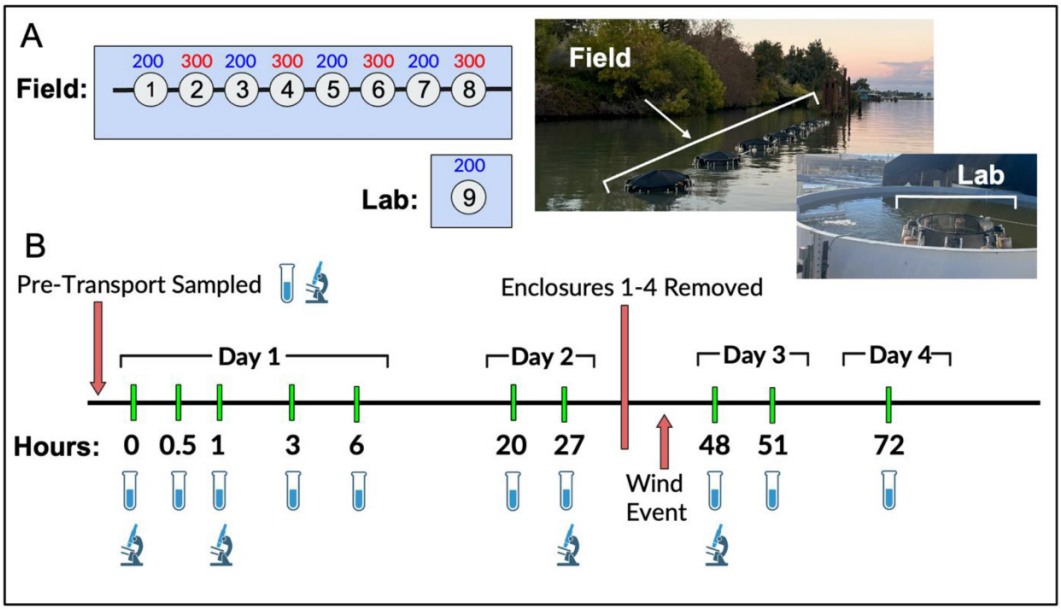
populations in the Great Lakes in North America were recovered following a successful population supplementation program (Welsh et al. 2020), as were the once extirpated Lahontan cutthroat trout (*Oncorhynchus clarkii henshawi*) in Pyramid Lake, USA (Al-Chokhachy et al. 2020). However, many programs 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), with no bias towards any specific taxa. Quantifying the magnitude and duration of the physiological stress response to supplementation can yield insight into how supplemented fishes will perform once released to the wild (Teixeira et al. 2007; Dickens et al. 2010; Schreck 2010; Sampaio and Freire 2016; Sokolova 2021). This understanding can therefore reveal mechanisms limiting success of fish supplementation programs and direct management efforts to improve their effectiveness.

Fishes undergoing supplementation may encounter stressors including physical handling, confined transport, and rapidly shifting water chemistry (Harmon 2009; Sampaio and 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 and 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 energy source (Barton and Iwama 1991; Wendelaar Bonga 1997; Barton 2000; Barton 2002; Acerete et al. 2004; Harmon 2009; Kumai et al. 2012). During this process, energy is reallocated away from nonessential processes, and rapid glucose metabolism provides fuel to tissues under increased cardiac output (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). Increased metabolism due to stress can increase blood pCO_2 and H^+ and decrease blood pH leading to metabolic acidosis (Wells et al. 1986; Ferguson and Tufts 1992; Cicia et al. 2012; Davison et al. 2023). This response can be particularly heightened for fish that demand access the surface to maintain buoyancy, and during hours of surface restriction (as is the case of confined transport) can lead to mortality (Kwan et al. 2026). Moreover, while O_2 is typically maintained with bubbling during confined transport, the respired CO_2 accumulates and decreases water pH (Paterson et al. 2003; Sampaio and Freire 2016); this induces respiratory acidosis and requires the active removal of H^+ (Evans et al. 2005; Tresguerres et al. 2023). Lastly, release to atmospheric-equilibrated water from high CO_2 low pH transport conditions can rapidly increase blood pH; this induces respiratory alkalosis and requires the rapid removal of HCO_3^- (Lonthair et al. 2020). Ionocytes (formerly called chloride cell and mitochondria-rich cell) are specialized cells designed to respond to IOA-B challenges (Evans et al. 2005; Tresguerres et al. 2023). The apical surface area of ionocytes can be remodeled to facilitate ion transport in response to IOA-B challenges, including ocean acidification (Kwan et al. 2024), harmful algal blooms (Skelton et al. 2024), salinity (Varsamos et al. 2002; Turko et al. 2012) and other stressors (Gilmour and Turko 2024), and this process is known to increase with cortisol circulation (Laurent and Perry 1990; Goss et al. 2001; Evans et al. 2005; Brannen and Gilmour 2018). As such, both the HPI axis and the gill ionocyte morphology responses can be informative for quantifying the stress incurred during fish transport and supplementation to enhance their establishment in their new environment. However, studies investigating these processes are rare and often not done in conjunction with active supplementation efforts that can maximize their application to real-world fish conservation.

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 their endemic habitat, the Sacramento-San Joaquin Delta (SSJD, CA, USA), including increased freshwater exports, competition from nonnative species, and intense drought (Hobbs et al. 2017; Moyle et al. 2016). 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 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 acclimatization enclosures designed to incubate them in the SSJD for several days before full release. Soft release strategies have been in use for decades and have grown in popularity in recent years. Studies on fish have shown that soft release improves survival and growth rate (Jonsson et al. 1999) and reduces atypical dispersal (Mokdad et al. 2022). Some recent studies have used physiological biomarkers to assess post-release recovery, but these studies are rare and results on the effect of soft release are mixed (Guest et al. 2017; Roberts et al. 2024). In a general review of soft release in wildlife reintroduction, soft-released animals were 1.47 times more likely to survive compared to hard-released animals (Tetzlaff et al. 2019). In studies specific to delta smelt, soft release has resulted in high survival, positive growth, transition to wild food sources, and has protected delta smelt from initial predation (Buckmeier et al. 2005; Baerwald et al. 2023; Gille et al. In Press). Effects of soft release enclosures vary by season

Fig. 1. Field and lab enclosure design and deployment locations, including density treatments (A) and sampling timeline (B). White brackets and arrows indicate enclosure placement in images. Enclosures in the field are numbered 1–8, and the lab enclosure is numbered 9. Blue and red numbers represent 200 (low density) and 300 (high density) fish per enclosure. Red lines and arrows denote the sampling point for the pre-transport group, when enclosures were removed, and when the wind event occurred. Green lines denote fish sampling timepoints. Test tube symbols represent time points that cortisol, glucose, and lactate were analyzed, and the microscope symbol represents time points gill ionocyte analysis occurred.



and location (Davis et al. 2024), and there is very little data on growth and survival for the hard release method. The two methods pose identical stressors to delta smelt—an initial period of intense handling followed by prolonged transport and translocation to the SSJD—which likely elicit equal stress responses. Yet, following release to the SSJD, hard release fish are expected to survive and reproduce without an opportunity for stress recovery.

We predict soft release could provide an opportunity for recovery from the multitude of stressors induced by the supplementation process that may negatively impact delta smelt performance and fitness in the wild. However, the magnitude of stress response, whether recovery occurs within enclosures, and after how long are unknown (Guest et al. 2017; Roberts et al. 2024). Here, we use "recovery" to denote stress response metrics that have declined from peak levels to values that are statistically indistinguishable from pre-transport conditions. Additionally, the optimal density at which to transport and enclose delta smelt to minimize the stress response and maximize cost-effectiveness is not understood (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 the stress response when using the soft release methodology at two density levels. We monitored water chemistry throughout the 2 h transport period and quantified trends in cortisol, glucose, lactate, and latent mortality following release into enclosures in the SSJD at 10 timepoints across 72 h. 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. These methods were replicated concurrently using a single enclosure deployed in a recirculating tank at FCCL to serve as a reference for environmental conditions in the field. Our study aims to inform ongoing delta smelt supplementation efforts by quantifying an appropriate acclimatization period to ensure stress recovery before release, testing for optimal transport densities, and providing a stress-response baseline for future release strategies. Ultimately, our goal is to improve the efficacy of this supplementation program by ensuring that delta smelt are released in a state of physiological homeostasis that promotes the expression of natural behaviors critical for survival and reproduction in the wild. We also aim for these results to inform supplementation strategies for other threatened and endangered fishes across the globe.

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 and Gille et al. In Press). Eight enclosures were deployed in the SSJD at the city of Rio Vista, CA, USA (38.1439, −121.6931) on 29 November 2022. Enclosures were connected in a line using steel cable and anchored in place by pylons (Fig. 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

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large recirculating tank (height: 1.5 m, width: 20 m) at FCCL as a comparison to conditions in the SSJD (Fig. 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

Due to the scale of effort and sensitivity of the species, the delta smelt supplementation program has rigid guidelines for handling and transport methodology (i.e., rearing conditions, handling, transport containers, density, water chemistry) that our experiment was explicitly designed to mimic. We did not seek to alter these methods to minimize stress during this experiment; rather, we aimed to capture the stress responses indicative of the response of delta smelt to these real-world supplementation conditions.

Delta smelt were fasted for 24 h prior to the experiment. Incoming SSJD water was used in FCCL for rearing and salinity was measured at 0.30 ppt by hatchery staff using a YSI PRO2030 (Xylem Inc., Washington DC, USA). On the day of the experiment, transport water was hyperoxygenated to ~19 $mg \cdot L^{-1}$ and salted to ~5.00 ppt with Instant Ocean (Spectrum Brands Family, Blacksburg, VA, USA). This is the common transport protocol at FCCL and is intended to reduce stress during transport (Cech et al. 1996; Swanson et al. 1996; Portz et al. 2006). Before being loaded with fish, transport water within carboys (75 L) was measured for temperature, dissolved O_2, and pH with a multiparameter YSI (650 MDS; Xylem Inc., 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 days post hatch, 69.0 ± 6.0 mm fork length, 2.5 ± 0.8 g; mean ± standard error of the mean, s.e.m.) were netted into nine carboys in total. Five carboys were filled with 200 fish each, and four carboys were filled with 300 fish each. These two densities were used to test the current agency transport method (300 fish per carboy) and a lower density that could reduce stress. Eight carboys (four with 200 fish, four with 300 fish) were transported by truck from FCCL to the SSJD (hereafter called "field"), loaded onto boats, and ferried to the enclosures. Approximately 2 h elapsed 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 the enclosure deployed in the recirculating tank at FCCL (hereafter called "lab"). Only one density treatment (200 fish) was represented at the lab site due to logistical constraints. 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.

Direct salinity measurements at the exact location of release were not available. To obtain an estimate of salinity at the time of release, conductivity and temperature data obtained from a California Department of Water Resources monitoring station 2 km upstream of the release location (CA Dept of Water Resources Station ID: RVB) was used to estimate salinity via a common conversion method (Hill et al. 1986). Using this method, salinity was estimated at 1.00 ppt at the time of release into field enclosures and fluctuated between ~2.00 and ~0.10 ppt for the duration of the study. At the lab location, salinity was measured at 0.30 ppt at the time of release to the lab enclosure.

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 $g \cdot L^{-1}$; Sigma–Aldrich, St. Louis, MO), and immediately frozen on dry ice to serve as a comparison to handling and transport stress (hereafter called "pre-transport"). Use of this buffered MS-222 solution throughout the study resulted in total euthanasia within ~30 s. This group cannot be considered a true "control" and instead serves as a useful reference for fish under minimal stress levels necessary for comparisons to post transport stress levels.

Starting at 11:20 AM on 29 November 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 (Fig. 1). As each carboy was emptied into an enclosure, eight fish were simultaneously netted as they swam out of the carboy and were euthanized with buffered MS-222 (0.5 $g \cdot L^{-1}$; Sigma–Aldrich, St. Louis, MO) then sampled. Five fish of the eight were measured for length (mm), 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. Eight fish were sampled immediately upon release into enclosures (hereafter called "0 h"), and after 0.5, 1, 3, 6, 20, and 27 h after release in all enclosures, and at each time point euthanasia and sampling took <5 min to complete. We note that the 0 h measurements represent the physiological state of the fish immediately following transport and prior to any stress recovery afforded by the enclosures. As such, they are a reasonable proxy for a hard release scenario, as hard release fish would be expected to enter the environment in this same physiological state without an opportunity to recover.

Release of fish at each field enclosure could not occur simultaneously. Fish were released in enclosure 1 at 11 am, enclosure 2 at 11:05, and so on. Therefore, sampling of each enclosure was also staggered in time by ~5 min to ensure consistency of each time point interval (i.e., 0.5, 1, 3 h after release) across enclosures. Removal of fish from the enclosures for sampling occurred via gentle physical netting with minimal disturbance to other fish in the enclosure. Our sampling 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

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nonvisible mortalities (submerged) were counted at the end of the experiment.

High winds forecasted for the evening following the 27 h time point were predicted to cause significant wave actions within the enclosures, potentially leading to high mortality. To maintain a reliable count of nonvisible 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 h sampling time point, mortalities were counted, and remaining fish were euthanized with buffered MS-222 (0.5 g·L^{-1}; Sigma–Aldrich, St. Louis, MO). 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 h (Fig. 1). This resulted in a halved sample size at each time point following the 27 h time point. After sampling at the 72 h time point, all remaining enclosures were removed from the field and lab site, mortalities were counted, and remaining fish were euthanized with buffered MS-222 (0.5 g·L^{-1}; Sigma–Aldrich, St. Louis, MO) and measured for length and weights.

All fish were collected, handled, and sampled under IACUC (Institutional Animal Care and Use Committee) protocol 23829. Permits for the collection, handling, and sampling of fish included recovery permit TE-027742-7 under the U.S. Endangered Species Act of 1973 and permit 2081a-2022-0011-R3 under the California Endangered Species Act of 1970. The euthanasia of delta smelt in this study was required under these permits to adhere to the genetic management program for delta smelt (Lindberg et al. 2013) that prohibits the release of fish designated for research purposes only.

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 five 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 s. 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. Two different resuspension volumes were used to ensure the resulting sample fell within the standard curve of the assay.

Cortisol analysis was conducted using an enzyme-linked immunosorbent assay (ELISA) kit (Salimetrics, Carlsbad, CA, USA) following the manufacturer's instructions. This kit was validated by Pasparakis et al. (2022) for use with whole-body delta smelt samples. Samples were run in duplicate and cortisol concentrations (ng·dL^{-1}) were calculated using four-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). Coefficient of variation was not allowed to exceed 5% for duplicate samples on the same plate. If this threshold was exceeded, the sample was re-extracted and assayed again to ensure data quality.

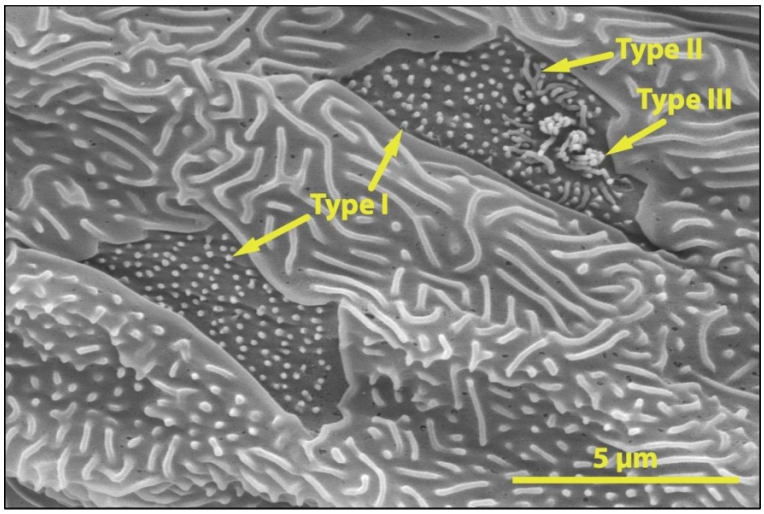
Metabolite extraction and assays

Metabolite (glucose and lactate) determinations followed methods in Bergmeyer (2012) 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 s. Two different elution volumes were used to ensure the resulting sample fell within the standard curve of the assay. Homogenates were then centrifuged at 10, 000 RCF for 10 min at 4 °C. Supernatants were removed and immediately neutralized with 54–58 μL of a 3 mol/L potassium carbonate solution (347825, Sigma–Aldrich, St. Louis, MO, USA), vortexed, and centrifuged again at 10 000 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 μg·g^{-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 (μg·mL^{-1}) was calculated from the absorbance values and was normalized for sample mass and expressed in μg·g^{-1} (μg of lactate per g of fish tissue).

Fig. 2. Scanning electron microscopy (SEM) image (25000x magnification) of gill ionocyte apical pit area and the three microvilli types observed and used in analysis. The apical pit is distinctly depressed and darkened in color relative to the primary gill surface. *Type I* microvilli are short and evenly distributed. *Type II* microvilli 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* microvilli.



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, and 48 h after release) due to cost and logistical constraints. 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* (Fig. 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,

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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.

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 transport 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. Post hoc pairwise contrasts were then evaluated for pairwise relationships among time point and density treatment and each water quality metric. The Bonferroni correction method was applied to resulting *p*-values to account for the simultaneous testing of multiple comparisons, α remained at 0.05.

To evaluate differences in cortisol, glucose, and lactate between time points (hours after release to enclosures) and the pre-transport 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 includes the pre-transport. 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 pre-transport 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 (all $p > 0.18$). These effects were removed from the models 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.

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), 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. A random intercept term was included for replicate enclosures. ANOVAs were run on each LMM to assess the relative significance of the effects. Post hoc contrasts were 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 pre-transport group. *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. Cortisol, glucose, and lactate data were log-transformed to meet model assumptions. All data are presented as 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

Transport water chemistry

From the start of carboy transport to the end, dissolved O_2 and pH significantly decreased across all carboys ($t = -2.87$, $p < 0.001$; $t = -23.12$, $p < 0.001$), and pCO_2 significantly increased across all carboys ($t = 14.12$, $p < 0.001$; Table 1). Dissolved O_2 and pH significantly decreased in both low- and high-density carboys (low: $t = 36.45$, $p < 0.001$, $t = 32.77$, $p < 0.001$; high: $t = 36.45$, $p < 0.001$, $t = 32.77$, $p < 0.001$), 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 (low: $t = -13.65$, $p = 0.001$, high: $t = -16.85$, $p < 0.001$) and the effect was 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 across all carboys ($t = -1.65$, $p = 0.15$; $t = -0.01$, $p = 0.99$) or between densities ($t = 0.70$, $p = 0.51$; $t = -0.03$, $p = 0.98$).

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 h 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 h time point), we observed 1.75% total

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).

Factor–Timepoint	Salinity (ppt)	Temperature (°C)	O_2 (mg·L^{-1})	pH	Alkalinity (mg·L^{-1})	pCO_2 (mm Hg)
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	1.00	10.50	10.96	7.85	68.00	317.40
Lab–Release	0.30	11.30	11.30	8.00	100.00	327.40

Note: Temperature, O_2, pH, and pCO_2 were measured in all nine carboys and alkalinity was measured in three 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 are presented as mean (s.e.m). Data for the field and lab environmental conditions were single measures.

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mortality in the low-density enclosures and 2.0% total mortality in the high-density enclosures (Table S1).

Cortisol

Cortisol levels varied significantly across hours after release to enclosures (ANOVA; $F = 19.31_{10,\,235}$, $p < 0.01$; Fig. 3A), 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 h mark when lab cortisol was higher (4.18 ± 1.21 ng·g^{-1}) than the field (1.53 ± 0.44 ng·g^{-1}; pairwise analysis; $t = -3.36_{123}$, $p = 0.01$).

In the field, cortisol increased ∼28x from pre-transport levels (0.38 ± 0.06 ng·g^{-1}) to a peak at 0.5 h after release (10.60 ± 1.42 ng·g^{-1}; Fig. 3A), and these levels were significantly elevated from the pre-transport (pairwise analysis; $t = -12.02_{46}$, $p < 0.01$). Cortisol levels significantly decreased from peak levels by 20 h after release (4.22 ± 0.85 ng·g^{-1}; pairwise analysis; $t = 3.82_{229}$, $p < 0.01$) but remained significantly elevated from the pre-transport until 72 h after release (1.22 ± 0.46 ng·g^{-1}; pairwise analysis; $t = -2.83_{67}$, $p = 0.34$).

In the lab, cortisol increased ∼22x from pre-transport levels to a peak at 0 h after release (8.29 ± 1.78 ng·g^{-1}), and this peak was significantly higher than the pre-transport (pairwise analysis; $t = 7.71_{69.3}$, $p < 0.01$). The general trend was for decreasing levels of cortisol in the lab, but from the 6 to 20 h mark cortisol increased (1.65 ± 0.28 ng·g^{-1} to 7.28 ± 2.97 ng·g^{-1}; 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 pre-transport levels by 48 h after release (0.71 ± 0.26 ng·g^{-1}; pairwise analysis; $t = 4.10_{228}$, $p < 0.01$; $t = 2.38_{88}$, $p = 1.00$).

Glucose

Glucose levels in delta smelt varied significantly across hours after release into enclosures (ANOVA; $F = 3.92_{10,59}$, $p < 0.01$; Fig. 3B). 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 increased to levels nearly double the pre-transport (63.16 ± 6.58 μg·g^{-1}) by 0.5 h after release (119.20 ± 10.17 μg·g^{-1}), but pairwise analysis showed this was not significantly higher than the pre-transport (pairwise analysis; $t = 3.93_{17}$, $p = 0.06$). Glucose after 1 h was very similar to the values at 0.5 h (117.26 ± 7.25 μg·g^{-1}) and pairwise analysis showed it was significantly higher than the pre-transport (pairwise analysis; $t = 4.07_{17}$, $p = 0.04$). Glucose was no longer significantly elevated from the pre-transport by 3 h after release (110.59 ± 8.45 μg·g^{-1}; pairwise analysis; $t = 3.52_{17}$, $p = 0.14$) and was significantly decreased from peak levels (pairwise analysis; $t = 5.09_{232}$, $p < 0.01$) and similar to pre-transport levels by 27 h after release (70.34 ± 46.57 μg·g^{-1}; pairwise analysis; $t = -0.57_{18}$, $p = 1.00$).

At the lab location, glucose increased to levels ∼1.5x the pre-transport by 0 h after release (101.00 ± 12.50 μg·g^{-1}), but this was not significantly higher than the pre-transport (pairwise analysis; $t = 2.17_{24}$, $p = 1.00$). No pairwise contrasts between hours after release in the lab were significantly different for glucose. From the 6–20 h mark in the lab, glucose showed a nonsignificant increase (52.20 ± 5.65 μg·g^{-1} to 87.55 ± 9.95 μg·g^{-1}; pairwise analysis; $t = 2.12_{232}$, $p = 1.00$).

Lactate

Lactate levels in delta smelt varied across hours after release into enclosures (ANOVA; $F = 2.80_{10,\,90}$, $p < 0.01$; Fig. 3C), and there was no interaction between hours after release and location (ANOVA; $F = 0.77_{10,\,241}$, $p = 0.65$). In the field, lactate increased to levels ∼1.4x pre-transport levels (398.19 ± 40.37 μg·g^{-1}) by 0.5 h after release (565.14 ± 36.88 μg·g^{-1}) but this increase was not significant (pairwise analysis; $t = -1.72_{10}$, $p = 1.00$). Lactate was not significantly different from the pre-transport at any other hour. Peak lactate was significantly decreased in field enclosures by the 6 h mark when lactate had fallen to levels below the pre-transport (355.28 ± 36.71 μg·g^{-1}; pairwise analysis; $t = 6.14_{241}$, $p < 0.01$). At the lab, lactate levels were increased at 0 h after release (471.10 ± 53.91 μg·g^{-1}), and this was not significantly different from the pre-transport (pairwise analysis; $t = -0.57_{12}$, $p = 1.00$). No other pairwise comparisons between hours in the lab were found to be significant.

Gill ionocyte morphology

Total gill ionocyte SA varied significantly across hours after fish were released into enclosures (ANOVA; $F = 5.82_{4,46}$, $p < 0.01$; Fig. 4) and was significantly affected by the

Fig. 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 pre-transport levels. Blue denotes data from the field and pink represent data from the lab. Blue letters (a, b, c, d, e, f) represent pairwise contrasts within the field location, and pink letters (v, w, x, y) indicate pairwise comparisons within the lab location. Differences in letters represent results from the pairwise testing. Asterisks (*) indicate significant differences between locations at individual time points using pairwise contrasts ($p < 0.05$). Points, error bars, and letters for the field and lab are jittered. Data are represented as mean ± s.e.m.

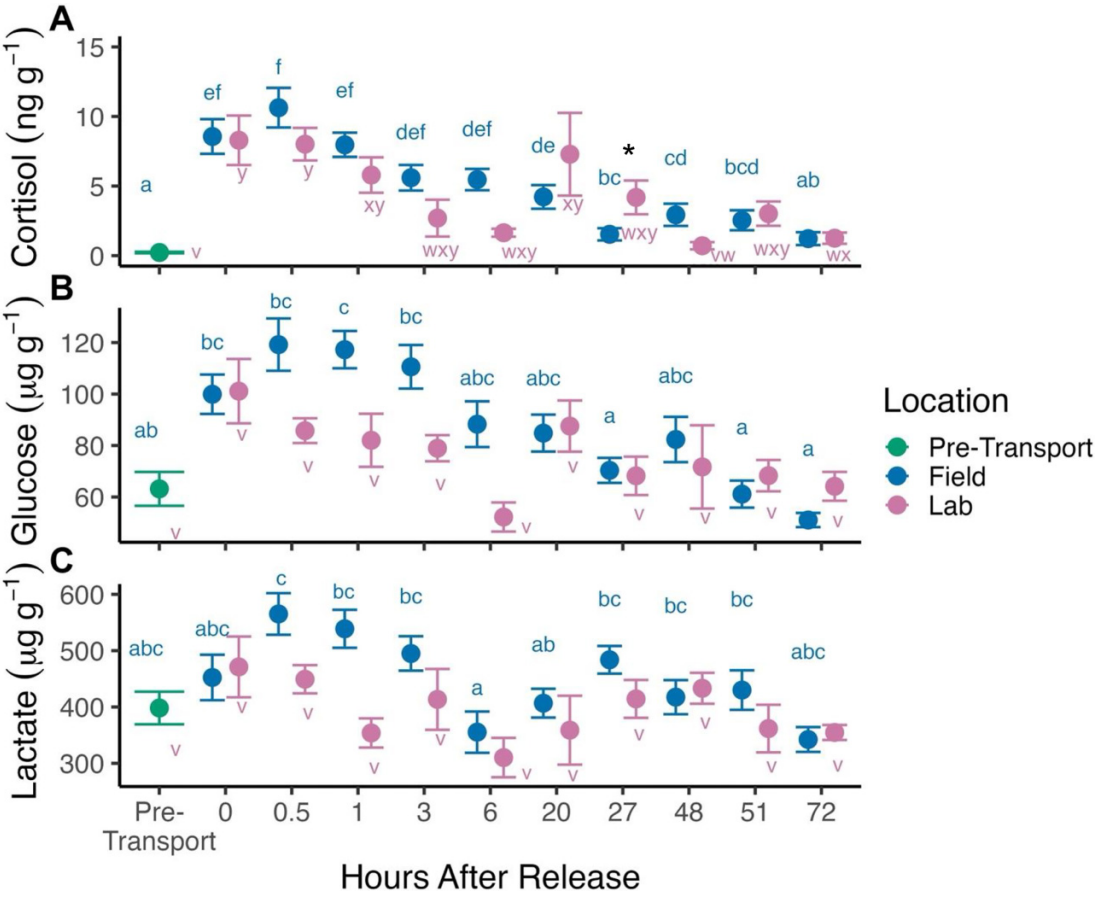
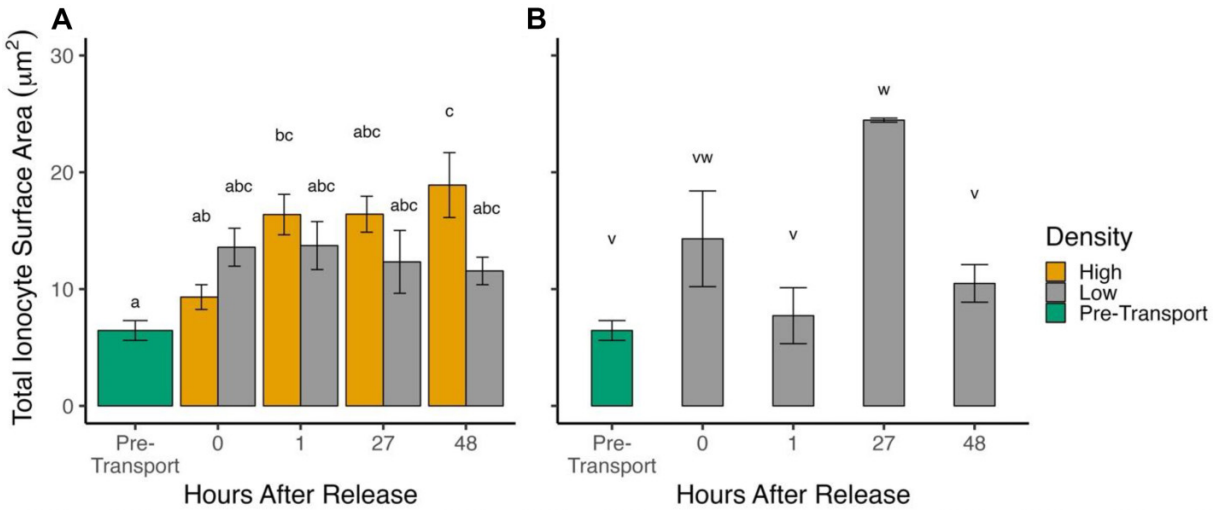
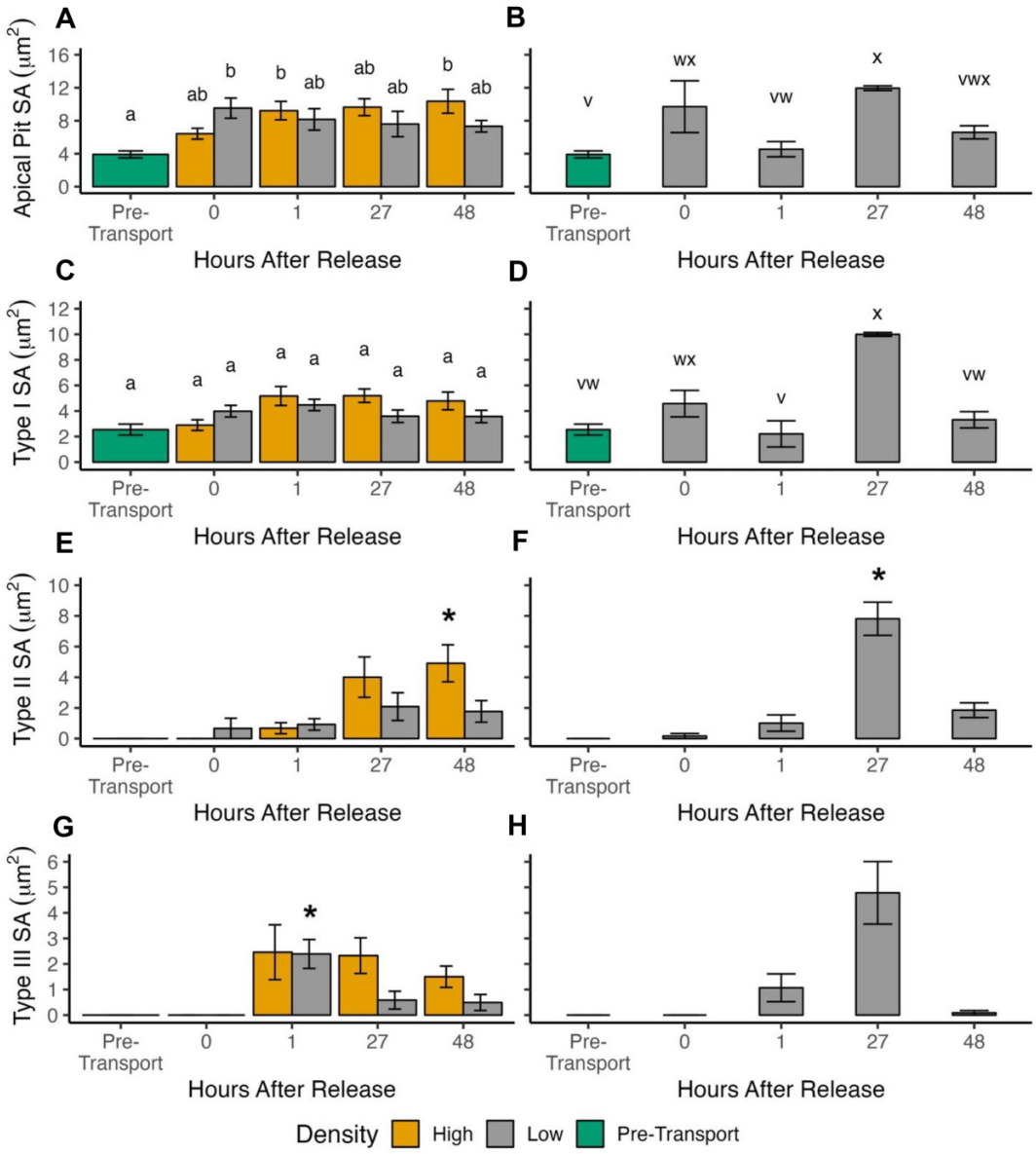


Fig. 4. Total gill ionocyte surface area (μm^2) across hours after release at the field location (A) and lab location (B). Orange represents data from high-density enclosures and gray represents data from low-density enclosures. Green represents the pre-transport group and is identical for both field and lab. Letters a, b, and c represent pairwise contrasts within the field location letters v and w indicate pairwise comparisons within the lab location. Data are represented as mean ± s.e.m.



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Fig. 5. Surface area (SA; μm^2) of apical pit, *Type I*, *II*, and *III* microvilli across hours after release at the field location (A, C, E, G) and lab location (B, D, F, H). Orange represents data from high-density enclosures, and gray represents data from low-density enclosures. Green represents the pre-transport group and is identical for both field and lab. Letters a and b represent pairwise contrasts within the field location and letters v, w, and x indicate pairwise comparisons within the lab location for apical pit and *Type I* microvilli (ANOVA). Asterisks indicate significant differences from zero for *Type II* and *III* microvilli at both locations. Data are represented as mean ± s.e.m.



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 pre-transport levels (6.23 ± 0.53 μm^2) by 1 h after release (16.55 ± 0.86 μm^2; pairwise analysis; $t = 3.90_{19}$, $p = 0.03$) and peaked to levels triple the pre-transport by 48 h after release (19.05 ± 1.10 μm^2; pairwise analysis; $t = 4.43_{19}$, $p = 0.01$; Fig. 4A). In the low-density enclosures in the field, total gill ionocyte SA at 1 h after release (13.66 ± 0.74 μm^2) was elevated but not significantly different from the pre-transport (pairwise analysis; $t = 3.09_{19}$, $p = 0.22$). In the lab, total gill ionocyte SA peaked to levels 4x the pre-transport by 27 h after release (24.46 ± 1.42 μm^2; pairwise analysis; $t = 4.96_{19}$, $p < 0.01$). When comparing across the low-density enclosures in the field and lab locations, total gill ionocyte SA was higher in the field at 1 h after release (13.66 ± 0.74 μm^2, 9.89 ± 1.18 μm^2; pairwise analysis; $t = 2.84_{19}$, $p = 0.04$) and was ~2x higher in the lab compared to field at 27 h after release (12.12 ± 0.89 μm^2, 24.46 ± 1.42 μm^2; 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 SA. ANOVA analysis revealed that apical pit and *Type I* microvilli SA varied significantly across hours after

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release to enclosures (ANOVA; $F = 5.37_{4,46}$, $p < 0.01$; $F = 5.82_{4,46}$, $p < 0.01$; Fig. 5). 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 pre-transport levels ($3.81 \pm 0.31\,\mu m^2$) by 1 h after release in high-density enclosures ($9.07 \pm 0.55\,\mu m^2$, pairwise analysis; $t = 3.87_{19}$, $p = 0.04$; Fig. 5A), and by 0 h after release in the low density enclosures ($9.20 \pm 0.55\,\mu m^2$, pairwise analysis; $t = 4.25_{19}$, $p = 0.02$). In the lab, apical pit area was significantly elevated from the pre-transport at the 0 h time point ($9.33 \pm 0.96\,\mu m^2$, pairwise analysis; $t = 4.42_{19}$, $p = 0.04$; Fig. 5B). Apical pit SA did not significantly differ between the field and lab locations at any hour 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 high and low densities in the field, *Type I* microvilli SA did not significantly differ from the pre-transport ($2.45 \pm 0.27\,\mu m^2$) despite values ~2x greater than the pre-transport at 1 h after release in high-density enclosures ($5.05 \pm 0.29\,\mu m^2$; pairwise analysis; $t = 2.22_{19}$, $p = 1.00$, Fig. 5C). In the lab, *Type I* microvilli SA was significantly elevated from the pre-transport by 27 h after release due to a peak 4x higher than the pre-transport ($10.02 \pm 0.46\,\mu m^2$; pairwise analysis; $t = 3.93_{19}$, $p = 0.01$; Fig. 5D). Between low-density enclosures in the field and lab, *Type I* microvilli SA was ~1.5x higher in the field than the lab at 1 h after release ($4.41 \pm 0.21\,\mu m^2$, $2.98 \pm 0.44\,\mu m^2$; pairwise analysis; $t = 3.81_{19}$, $p < 0.01$) and was ~3x less in the field than lab at 27 h after release ($3.58 \pm 0.24\,\mu m^2$, $10.02 \pm 0.46\,\mu m^2$; pairwise analysis; $t = -3.12_{21}$, $p = 0.02$).

No *Type II* microvilli were observed in the pre-transport samples, as such a value of $0.00\,\mu m^2$ was used to represent the pre-transport levels (see Materials and Methods). Average *Type II* microvilli SA gradually increased from ~1.00 to ~5.00 μm^2 across the experimental period. In low-density enclosures in the field, *Type II* microvilli SA was not significantly elevated from pre-transport levels at any hour after release (Fig. 5E). In high-density enclosures in the field, *Type II* microvilli SA was significantly elevated from the pre-transport by 48 h after release ($5.22 \pm 0.48\,\mu m^2$; one-sample *t*-test; $\mu_0 = 0$; $t = 4.07_5$, $p = 0.03$; Fig. 5E). In the lab, *Type II* microvilli SA was significantly elevated from pre-transport levels at 27 h after release ($8.03 \pm 0.53\,\mu m^2$; one-sample *t*-test; $\mu_0 = 0$; $t = 7.20_{12}$, $p = 0.04$; Fig. 5F).

No *Type III* microvilli were observed in the pre-transport samples, or at 0 h after release. As such, a value of $0.00\,\mu m^2$ was used to represent the pre-transport levels (see Materials and methods). *Type III* microvilli SA was $2.46 \pm 1.07\,\mu m^2$ in high-density enclosures and $2.39 \pm 0.57\,\mu m^2$ in low-density enclosures in the field at 1 h after release and decreased gradually to $1.50 \pm 0.42\,\mu m^2$ and $0.49 \pm 0.31\,\mu m^2$, respectively, by 48 h after release. In low-density enclosures in the field, *Type III* microvilli SA was significantly elevated from pre-transport levels at 1 h after release ($2.39 \pm 0.57\,\mu m^2$; one-sample *t*-test; $\mu_0 = 0$; $t = 4.22$, $p < 0.03$; Fig. 5G), high-density enclosures were never significantly elevated. In the lab, *Type III* microvilli SA was never significantly elevated from the pre-transport (Fig. 5H).

Discussion

Fish supplementation is a pervasive management strategy aimed at conserving dwindling wild populations and re-establishing extirpated species (Armstrong and Seddon 2008), yet many supplementation programs fail to increase their respective population size or establish self-sustaining populations (Cochran-Biederman et al. 2015). Physiological stress incurred during transport and release likely inhibits performance, survival, and reproduction of released fishes in the wild, and may ultimately limit the effectiveness of supplementation programs (Barton and Iwama 1991; Barton 2000; Harmon 2009; Dickens et al. 2010; Sampaio and Freire 2016; Zimmer and Perry 2022). Our study was aimed at improving the efficacy of an active supplementation program for the critically endangered delta smelt by quantifying the magnitude and duration of their stress response when utilizing novel acclimatization enclosures. We were successful in identifying an appropriate enclosure acclimatization period that ensures recovery from elevated stress response metrics. This acclimatization period is therefore likely to promote the expression of natural behaviors critical for survival and reproduction in the wild.

Our results demonstrate that delta smelt experience significant physiological stress 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; Milligan 2003; Lankford et al. 2005; Carbonara et al. 2010), predator avoidance (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; Campbell et al. 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 and glucose in delta smelt following release to enclosures, indicating that a substantial stress response was elicited from supplementation methods. Lactate was elevated following release, but this increase was not significantly different from pre-transport levels. 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

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cortisol and the presence of novel microvilli types, and their differential usage suggests they may serve distinct functions. To the best of our knowledge, this study is the first to report individual gill ionocytes having multiple types of microvilli on their apical surface. Our results indicate that recovery of cortisol, glucose, and lactate is achieved after 72 h 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 h in sea raven *Hemitripterus americanus* (Vijayan and Moon 1994). The magnitude of plasma and whole-body cortisol increase following stressors varies 100x (~3–300 ng·mL^{-1}) across teleost and elasmobranch fishes and the typical recovery time to baseline levels occurs anywhere from 2 to 48 h (reviewed in Barton 2002 and Pankhurst 2011).

In a previous experimental study that quantified the stress response of delta smelt, whole-body cortisol peaked to levels ~20x the control after 15–30 min following an acute netting and air exposure treatment (Pasparakis et al. 2022). In a study of juvenile spotted sea trout (*Cynoscion nebulosus*) exposed to handling and transport to acclimation enclosures, tissue cortisol peaked 6x from baseline levels immediately after transport (Guest et al. 2017). Comparatively, the ~28x increase in whole-body cortisol observed here by 30 min after release likely reflects the accumulation of stressors faced by delta smelt during full-scale supplementation. The increase in whole-body cortisol levels relative to the pre-transport observed here is high compared to most studies measuring plasma cortisol following 2 h transport periods (reviewed in Sampaio and Freire 2016). For example, in a study on four trout species following acute handling and 2 h transport, peak plasma cortisol was reached by 3 h after release and was 30–40x higher than the control (Barton 2002). Roberts et al. (2024) assessed plasma cortisol in soft-released Atlantic salmon and found a 4–5x increase from control levels following release to enclosures, but no significant recovery in enclosures. While we did not measure extraction efficiency, the use of whole-body cortisol analysis likely resulted in lower extraction efficiency compared to plasma cortisol analysis (Yeh et al. 2013). This discrepancy may exaggerate the differences observed in cortisol measurements relative to plasma-based studies, precluding direct comparisons to such studies.

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 cues, tidal cycles, changes in turbidity, or movement of enclosures could have increased cortisol in the field and hampered recovery (Hasenbein et al. 2013; Pasparakis et al. 2022). Environmental salinity in the lab was lower (0.30 ppt) than in the field (1.00 ppt) at the time of release. High salinity has been shown to reduce cortisol expression and improve survival during transport of delta smelt and other species (Cech et al. 1996; Swanson et al. 1996; Portz et al. 2006). However, field salinity levels fluctuated with the tidal cycle, with values as low as 0.1 and as high as 2.0. This fluctuating salinity condition may have served as another novel environmental stressor (along with predator cues, changes in turbidity, and movement of enclosures) that hampered stress recovery relative to the lab fish, especially since these fish had been acclimatized to stable 0.30 ppt in a hatchery environment (Komoroske et al. 2016; Evans and Kültz 2020). As such, the benefit of higher environmental salinity at the time of sampling for fish in the field is likely diluted by the stressors of being in a novel environment.

For fish in the lab, cortisol rapidly increased at the 20 h mark, then decreased back to baseline levels by 48 h. Shortly before sampling at 20 h 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, 2024). The noise and vibrations associated with this pumping could have led to an increase in cortisol at the 20 h 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 h) 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 cortisol results 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. Significant reduction of peak cortisol levels occurred as soon as 20 h after release, and cortisol recovered to pre-transport levels by 72 h after release. These results in combination with minimal mortality indicate that this stress response is within the allostatic capacity of delta smelt when using the soft release methodology. Cortisol results from the 0 h time point represent the condition of fish in the hard release methodology and suggest that hard release will result in poor fish performance due to the negative impact of high stress levels on adaptive behaviors and processes necessary for survival in the wild. In contrast, the use of in situ enclosures for delta smelt alleviates the negative impact of stress by allowing time for stress recovery and could result in higher expression of adaptive behaviors, such as schooling, feeding, predator avoidance, survival, and site fidelity as has been shown in other studies on in situ acclimatization (Jonssonn et al. 1999; Kuwada et al. 2004; Brennan et al. 2006; Baerwald et al. 2023). Therefore, our data

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suggest that soft release supplementation should allow for at least 20 h of enclosure acclimatization, and future research should seek to elucidate sub-lethal impacts of increased cortisol on performance and fitness after release from enclosures.

Glucose and 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; Acerete et al. 2004; Jiang et al. 2017; Pasparakis et al. 2022). 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 Freire 2016). Elevation of glucose and lactate following a stressor is typically delayed and proceeds the circulation of cortisol (Wendelaar Bonga 1997; Mommsen et al. 1999; Barton 2002), yet we observed the simultaneous elevation of all three metrics at the first sampling event. Handling and netting of delta smelt began approximately 2 h prior to the first sample and likely activated the HPI axis and led to elevated glucose and lactate prior to the first sampling event. This may explain the simultaneous elevation of all three metrics.

Our data suggest that mobilization of glucose was needed to fuel increased energy use in delta smelt across the supplementation process. Stress does not always result in increased metabolism; however, the significant elevation of cortisol and glucose suggests energy mobilization was occurring in this case, which may indicate increased metabolism (Wendelaar Bonga 1997; Mommsen et al. 1999; Schreck and Tort 2016). Although lactate did not reach levels that were statistically significant from pre-transport levels, the increase of ~37% relative to pre-transport in conjunction with increases in cortisol, as can occur during HPI axis responses (Milligan and Girard 1993; Wendelaar Bonga 1997; Jiang et al. 2017), suggests that O_2 demand may have exceeded supply in some tissues leading to anaerobic metabolism and the accumulation of lactate. In Pasparakis et al. (2022), 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 Roberts et al. (2024), Atlantic salmon showed an ~1.5x increase in concentrations of plasma glucose and lactate compared to the control following transport and demonstrated recovery after 2 days of soft release acclimatization. In Eurasian perch (*Perca fluviatilis*) and various trout species subjected to handling and transport for 2–4 h, researchers observed a 2x elevation in glucose but no increase in lactate (Barton 2002; Acerete et al. 2004). The relatively greater increase in lactate observed here compared to these studies could indicate a greater degree of anaerobic metabolism characteristic of intense stress or exercise (Milligan and Girard 1993) but may also be an artifact of our whole-body analysis method and reduced extraction efficiency. These results underscore the magnitude of metabolic demand delta smelt experience during the transport and release process. However, our data show that glucose levels both significantly declined from peak levels and returned to pre-transport levels by 27 h after release, and lactate returned to levels slightly below the pre-transport by 6 h after release.

In conjunction with cortisol, glucose and lactate increased at the 20 h mark in the lab before decreasing back to baseline levels. This could have been in direct response to 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 mobilized at least a portion of 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 recover from elevated glucose and lactate levels during soft release (Van Ginneken et al. 2008; Schreck 2010). 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.

Our metabolite results agree with other studies on soft release (Roberts et al. 2024) suggesting that the use of the soft release methodology, opposed to hard release, allows for an important period of recovery from elevated metabolite levels. This acclimatization 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*b*) 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. 1992*a*; Perry and Goss 1994; Furukawa et al. 2011; Dymowska et al. 2012; Kwan et al. 2024). Similar to cortisol, glucose, and lactate responses, the general pattern for total ionocyte SA response includes an initial increase that dampens over time. However, the total ionocyte SA remained relatively higher than pre-transport levels throughout the experimental period. This suggests the delta smelt can benefit from the time provided during soft release to adjust their gill IOA-B

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mechanisms. Gill remodeling would presumably require energy, but whether it represents a significant compromise is not known. However, recent advances on delta smelt respirometry now allows for the assessment of sublethal stressors (Kwan et al. 2026)—and in this instance, the rapid adjustment of acid-base and salinity conditions. Regardless, future research on the relationship between delta smelt ionocyte ultrastructure and their environment may allow conservation managers to prime delta smelt within the hatchery for more optimal release and recovery of their populations.

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. 1992*a*; Hwang 2009; Furukawa et al. 2011): gill ionocytes usually exchange environmental Na^+ for plasma H^+ to normalize blood pH levels, leading to an accumulation of plasma HCO_3^-. The rate of ion exchange would not only be expedited by increased ionocyte apical SA and *Type I* microvilli, but also by the greater NaCl available due to salt addition (5.00 ppt; Table 1). We observed an increase in apical area and *Type I* microvilli directly following release (0 h after release) consistent with known mechanisms of acidosis recovery. The lack of *Type II* and *Type III* microvilli in the pre-transport and upon release (0 h after release), and their differential expression over time, indicate that these structures may serve a different purpose than the apical area and *Type I* microvilli. Past studies have consistently reported a single type of microvilli within gill ionocytes (Franklin 1990; Brown 1992; Goss et al. 1994; Varsamos et al. 2002; Fridman et al. 2011; Kwan et al. 2019, 2024; Skelton et al. 2024); consequently, our discovery of three different microvilli types expressed 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 (0.20 ppt) European seabass (*Dicentrarchus labrax*). Regardless, this study is the first to 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^- using expanded ionocyte surface area. 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 or directly following release to enclosures (0 h after release) but were in much greater abundance by 1 h of enclosure acclimatization when HCO_3^- removal was likely occurring. Moreover, fish released to the field were exposed to 5x less salinity than they experienced during transport (Carboy: 5.00 ppt, Field: 1.00 ppt). As such, the significantly less NaCl in the field likely slowed IOA-B regulation. In contrast, fish released into the lab were exposed to 16.7x less salinity (Carboy: 5.00 ppt, Lab: 0.30 ppt, Table 1). Although the lab fish were exposed to lower salinity upon release, they showed a generally lower increase in total ionocyte SA, *Type II* and *Type III* microvilli than field fish. This counterintuitive finding is likely explained by the greater HPI axis activation in response to a novel environment, and the fluctuations in salinity for the field fish, both of which could have contributed to higher ionocyte expression (Laurent and Perry 1990; Goss et al. 1994, 2001; Varsamos et al. 2002; Brannen and Gilmour 2018). Fluctuating salinity could have been particularly stressful due to the novelty of these conditions for delta smelt that are reared at a relatively stable salinity of 0.30 ppt at FCCL. Greater understanding of the relationship between ionocyte ultrastructure with their environment may allow conservation managers to prime delta smelt within the hatchery for more optimal release.

Although additional experiments will be needed to confirm the role of *Type II* and *Type III* microvilli during the enclosure acclimatization period, their differential pattern hints at their roles in IOA-B regulation in a novel environment. 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 more variable salinity environment relative to stable rearing (0.30 ppt) and transport conditions (5.00 ppt). In contrast, the use of *Type III* microvilli peaked 1 h after release to field enclosures but dampened over time. This timeline would match the need for plasma HCO_3^- removal and acid-base recovery directly following transport. Finally, the magnitude of the *Type II* and *Type III* microvilli responses match the pattern seen with total ionocyte surface area: with the exception of the 27 h 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. This is likely due to the greater need for IOA-B regulation in response to higher stress levels in a fluctuating salinity environment in the field.

Cortisol is not only a stress response hormone but has also been linked to ionocyte responses and has been called the "saltwater adapting hormone" (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. 1983). 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; Wilson et al. 2002; Brannen and Gilmour 2018). Our results indicate that peak cortisol at 0.5 h after release to the field enclosures could have been partially in response to rapidly shifting water chemistry shortly after transport and corresponds with the significant increase in ionocyte surface area 1 h after release to enclosures, especially in the field enclosures. Moreover, upon the brief increase in cortisol at 27 h after release in enclosures in the lab, we observed a similar sudden increase in ionocyte surface area.

Together, our gill ionocyte morphology results indicate delta smelt mounted a considerable IOA-B regulatory response to shifting water chemistry across transport and release into the SSJD. Our observation of novel ionocyte morphology provides further lines of evidence that the response

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of delta smelt to supplementation stressors is substantial and potentially unique to that of frequently supplemented fishes, such as salmon, that are often used as a model for supplementation methodology. We believe these findings contribute to the multiple lines of evidence suggesting that minimizing stress during supplementation is an important management consideration to enhance the success of the supplementation program. Recommendations for future supplementation include less intense handling and utilizing transport containers that can off-gas CO_2 to limit acidosis during transport. We also recommend, to the extent possible, matching rearing conditions, such as salinity, to conditions in the wild, whether that be through acclimation in the laboratory prior to release or choosing release locations that better match hatchery conditions.

Conclusion

Our results demonstrate that the suite of stressors associated with delta smelt supplementation, including handling, transport, and rapid water chemistry transitions, elicit a measurable physiological stress response, particularly in cortisol and glucose levels. Following peak levels at 0.5 h after release, cortisol approached pre-transport values by 27 h of enclosure acclimatization and recovered completely by 72 h. Glucose recovered from peak levels at 1 h after release by 20 h of acclimatization. This indicates that soft release enclosures provide sufficient time for delta smelt to recover from elevated stress metrics following significant stress exposure. Although acute stress responses are adaptive, chronic or sustained stress can impair key behaviors and physiological functions, including feeding, predator evasion, schooling, and reproduction, which are critical for performance and survival in the wild (Wendelaar Bonga 1997; Teixeira et al. 2007; Dickens et al. 2010; Schreck 2010; Sokolova 2021). Soft-release enclosures may therefore enhance the successful transition of delta smelt to the SSJD by allowing recovery from prolonged stress induced by handling and transport, which could otherwise be exacerbated or unresolved following immediate release without acclimatization. In contrast, hard release—represented by the 0 h data—results in fish being released in a highly stressed state that may constrain adaptive behaviors and performance upon entry into the SSJD.

We recommend a minimum of 20 h of acclimatization in soft release enclosures prior to release as a practical guideline for future supplementation efforts. While direct post-release monitoring was not possible in this study, the physiological benchmarks established here provide a valuable foundation for optimizing delta smelt conservation strategies and informing conservation strategies for other threatened and endangered fishes. Future research should aim to pair physiological assessment with survival and behavioral outcomes following release to the wild.

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Data availability

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Competing interests

The authors declare there are no competing interests.

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Supplementary material

Supplementary data are available with the article at https://doi.org/10.1139/cjfas-2024-0390.

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