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Title

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Permalink

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Journal

Comparative Biochemistry and Physiology Part A Molecular & Integrative Physiology,
274

ISSN

1095-6433

Authors

Pasparakis, Christina
Wampler, Alexandra N
Lohroff, Toni
[et al.](#)

Publication Date

2022-12-01

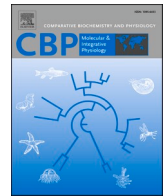
DOI

10.1016/j.cbpa.2022.111303

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Peer reviewed



Characterizing the stress response in juvenile Delta smelt exposed to multiple stressors

Christina Pasparakis^{a,b,*}, Alexandra N. Wampler^c, Toni Lohroff^{c,d}, Francine DeCastro^d, Dennis E. Cocherell^c, Evan W. Carson^e, Tien-Chieh Hung^f, Richard E. Connon^g, Nann A. Fangue^c, Anne E. Todgham^d

^a Department of Environmental Toxicology, University of California Davis, Davis, CA, USA

^b Bodega Marine Laboratory, University of California Davis, Bodega Bay, CA, USA

^c Department of Wildlife, Fish and Conservation Biology, University of California Davis, Davis, CA, USA

^d Department of Animal Science, University of California Davis, Davis, CA, USA

^e U.S. Fish and Wildlife Service, San Francisco Bay-Delta Fish and Wildlife Office, Sacramento, CA, USA

^f Fish Conservation and Culture Laboratory, Department of Biological and Agricultural Engineering, University of California Davis, Davis, CA, USA

^g School of Veterinary Medicine, Department of Anatomy, Physiology and Cell Biology, University of California Davis, Davis, CA, USA

ARTICLE INFO

Editor: Michael Hedrick

Keywords:

Delta Smelt
Stress
Predation
Climate change

ABSTRACT

The Delta Smelt (*Hypomesus transpacificus*), once an abundant fish endemic to the Sacramento-San Joaquin Estuary, is now on the brink of extinction. Due to the high sensitivity of this species, knowledge of their stress response will be vital to their future survival and sustainability. Understanding the magnitude and kinetics of cortisol induction in Delta Smelt will provide valuable information when interpreting the degree of environmentally relevant stressors, such as warming and predator exposure. As little is known about the primary stress response and cortisol dynamics in Delta Smelt, the first aim of this study was to measure basal and maximal whole-body cortisol prior to and following exposure to a sublethal and significant netting stress at 17 and 21 °C. Our findings reveal that juvenile Delta Smelt held at 21 °C display an exacerbated stress response and a reduction in available energy compared to fish held at 17 °C. There was no evidence of the secondary stress response to the netting stress as whole-body glucose and lactate levels in treatment groups remained similar to basal values. The second aim of this study was to investigate the effect of a largemouth bass (*Micropterus salmoides*) predator cue, which was found to induce a significant increase in cortisol relative to control levels in juvenile Delta Smelt. Indices such as cortisol can be used as bioindicators of stress in the field and results from this study suggest that moderate temperatures and reduced predation are optimal release conditions during hatchery-based supplementation to minimize stress to this highly sensitive species.

1. Introduction

The stress response is an organism's reaction to a stimulus which is perceived as stressful or hazardous, and the resulting regulatory mechanisms elicited to return them to their normal homeostatic state (Pickering, 1981). A common misconception is that stress is exclusively detrimental to fish. In actuality, the stress response is an adaptive mechanism that allows an organism to maintain homeostasis while coping with perceived stressors and can be beneficial to fish by inducing their flight or fight response and prompting their escape from harmful

situations (Barton, 2002). Energy metabolism is central to the stress response. The amount of available energy, the rate of its assimilation and transformation, and the capacity of energy storage will ultimately set an organism's tolerance limits to environmental change (Sokolova et al., 2012).

Depending on the severity and duration, stressors may become detrimental or even fatal when an organism's physiological response mechanisms are compromised due to significant rise in energy demand and subsequent decline of aerobic scope (Pörtner, 2001, 2010). Maintaining homeostasis during stress can be energetically expensive and its

* Corresponding author at: Department of Environmental Toxicology, University of California Davis, Bodega Marine Laboratory, 2099 Westshore Rd, Bodega Bay, CA 94923, USA.

E-mail address: cpasparakis@ucdavis.edu (C. Pasparakis).

<https://doi.org/10.1016/j.cbpa.2022.111303>

Received 18 May 2022; Received in revised form 16 August 2022; Accepted 24 August 2022

Available online 29 August 2022

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demand may exceed an organism's energetic limits. Organisms under chronic stress will have less energy available to allocate to basic physiological needs such as metabolism, growth, and reproduction (Kooijman, 2010). If stressors become too severe or long-lasting, they can lead to whole-body consequences, such as reduced growth, fecundity and immunity, that can ultimately cause significant reductions in fish populations and potentially result in ecosystem-level effects (Schreck, 2010; Tort, 2011; Wendelaar Bonga, 1997).

Stress physiology and the dynamics of endocrine control have been extensively studied in teleost fishes (Barton and Iwama, 1991; Iwama et al., 1997; Pankhurst, 2011; Pickering, 1998). Cortisol, the primary glucocorticoid in teleosts, is under control of the hypothalamus-pituitary-interrenal (HPI) axis and is released into circulation from interrenal tissue following the perception of stress (Mommensen et al., 1999). Increased circulating cortisol during the primary stress response promotes the mobilization of energy reserves to help fish cope with the increased energy demand associated with stressor exposure (Das et al., 2018). Metabolites such as glucose and lactate significantly increase in circulation after stressor exposure and are common indicators of the secondary stress response in fishes (Barton and Iwama, 1991). Metrics of the generalized stress response can be used as bioindicators of stress in wild populations as long as preliminary work is done to provide species specific information about cortisol dynamics (magnitude, duration, and recovery) and secondary physiological changes caused by stress under controlled laboratory conditions (Barton, 2002).

The Delta Smelt (*Hypomesus transpacificus*), once among the most abundant fish to inhabit the Sacramento-San Joaquin Delta, is now threatened by extinction (Bennett, 2005; Moyle et al., 2018). Delta Smelt underwent significant declines in their population starting in the early 1980s, with all trends pointing downward since 2002 (Hobbs et al., 2017; Thomson et al., 2010), and with fish being virtually undetectable in surveys since 2012 (Moyle et al., 2019). Due to these rapid declines, Delta Smelt were listed as threatened under both the California Endangered Species Act (CESA) and the Federal Endangered Species Act (FESA) in 1993 (U.S. Office of the Fed. Regist., 1993). Their status was escalated to endangered under the CESA in 2009, and they were classified as critically endangered by the International Union for Conservation in Nature Red List of Threatened Species in 2014 (CFGC, 2009; IUCN, 2015). The UC Davis Fish Conservation and Culture Laboratory (FCCL) was founded in 1996 with the purpose of rearing a captive population of Delta Smelt as a safety guard against extinction and for research purposes (Baskerville-Bridges et al., 2005; Lindberg et al., 2013). In 2008, the FCCL collaborated with UC Davis Genomic Variation Laboratory to establish a genetically managed refuge population (Fisch et al., 2013). Plans for supplementation of the wild Delta Smelt population are currently underway, and the first experimental release was conducted between December 2021 and February 2022 (USFWS, 2019; 2020). To maximize successful supplementation efforts, physiological research is used to inform rearing, transport, and release strategies that minimize stress and mortality in Delta Smelt (Lessard et al., 2018; Swanson et al., 1996).

The ultimate cause of the Delta Smelt decline is not isolated to one specific reason, but rather due to a combination of anthropogenically induced effects that have irreversibly changed the ecosystem in which they live (Brown et al., 2013, 2016; Moyle et al., 2016, 2018). Shifts in temperature, salinity, and turbidity due to both climate change and increased water diversion have significantly altered the Delta ecosystem, creating an environment less suitable for Delta Smelt and more suitable for invasive species such as the overbite clam (*Potamocorbula amurensis*) and warm water fishes such as the largemouth Bass (*Micropterus salmoides*) (Baumsteiger et al., 2017; Komoroske et al., 2015; Moyle et al., 2016). Increased predation and competition for food and resources due to the introduction of invasive species are major threats to the future sustainability of Delta Smelt in the wild. In addition, evidence suggests that Delta Smelt are already living close to their thermal capacity (Komoroske et al., 2014; Swanson et al., 2000).

Therefore, knowledge of how variables such as elevated temperature and predation affect the stress response in Delta Smelt is invaluable when considering rearing conditions in captivity and release conditions for future supplementation that minimize stress in this highly sensitive species.

There is a paucity of data about the kinetics and magnitude of cortisol induction in Delta Smelt. To reliably use cortisol as a bio-indicator of stress in both wild and cultured populations, a more comprehensive understanding of the generalized stress response is needed. The first aim of this study was to gain a better understanding of the stress response in juvenile Delta Smelt by investigating basal and peak cortisol levels, as well as the timing of cortisol response, following a common sublethal stressor in fish culture (i.e. netting). We investigated cortisol dynamics and two secondary response variables, whole-body glucose and lactate, in juvenile Delta Smelt held at two environmentally relevant temperatures, 17 and 21 °C, and following exposure to a severe but sublethal netting stress. The second aim of this study was to examine cortisol and secondary stress response variables resulting from an ecologically relevant predation stress. Following trials aimed to identify basal and maximal cortisol induction, Delta Smelt were exposed to a largemouth bass predator cue to test if a) this cue elicited a stress response and b) to examine the magnitude of this response relative to basal and induced peak cortisol levels. Characterizing the stress response in Delta Smelt will help clarify the difference between perceived benign vs. stressful conditions in this species. This, in turn, can be used not only to help elucidate the conditions best suited for the successful culture and supplementation of Delta Smelt, but can also allow researchers to more accurately characterize stressors in their natural environment.

2. Methods

2.1. Study species and maintenance

Juvenile Delta Smelt (*Hypomesus transpacificus*) (46.1 ± 0.2 mm fork length, 0.54 ± 0.01 g) were acquired from the University of California Davis Fish Conservation and Culture Laboratory (FCCL) in Byron, CA, USA. Specific details about the spawning and rearing of Delta Smelt and the design of the research facility are described in Lindberg et al. (2013), Tigan et al. (2020) and Hung et al. (2021). In October 2020, Delta Smelt were transferred from FCCL to the UC Davis Putah Creek Aquaculture Facility (PCF; Davis, CA) at ~160 days post-hatch (dph). Upon arrival, fish were immediately separated into 36, 15-gal polyethylene black tubs (referred to herein as sub tanks). Black baskets made with plastic mesh and built to fit snugly on the bottom of sub tanks were inserted prior to the introduction of Delta Smelt. Circular lids made from shade cloth were placed on individual sub tanks to reduce debris contamination, manage light intensity, and prevent fish from escaping. The PCF contains three parallel recirculating aquaculture systems with four 400-L tanks (referred to herein as holding tanks) in each system. Each system has its own temperature control unit. The holding tanks served as experimental water baths, and each holding tank contained 3 sub tanks, with 23 fish per sub tank (Supp. Fig. 1). An additional circular lid made from Styrofoam covered each holding tank to reduce stress to fish.

To maintain water quality, sub tanks were running flow through with pretreated clean water from outdoor reservoir tanks. Excess tank water overflowed through a 1-in. hole covered by mesh to prevent fish from escaping. Salinity was maintained at 2 ppt by adding Instant Ocean (Aquarium Systems, Mentor, OH) to outdoor reservoir tanks. Each sub tank had its own external biofiltration unit. Water in the sub tanks was pushed through the unit filled with k1 biomedica and returned to the tanks using an airlift mechanism. The airlift unit also maintained the oxygen level in the sub tanks.

Delta Smelt were acclimated to ambient conditions of 16 °C and 2 ppt for two weeks prior to the start of experiments (Supp. Table 1.) Salinity was set to reflect conditions where juvenile Delta Smelt are most

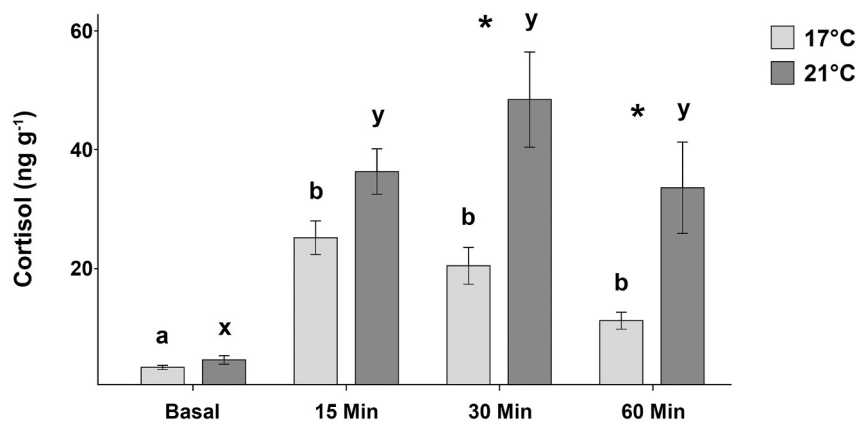


Fig. 1. Whole-body cortisol measurements (ng g^{-1}) in juvenile Delta Smelt prior to and following (15, 30, and 60 min) exposure to a sublethal netting stress expected to induce maximal cortisol levels conducted at 17 °C ($n = 12\text{--}35$) and 21 °C ($n = 12\text{--}35$). Data are presented as mean $\pm$ SEM and different letters represent significant differences between sampling times within a temperature and asterisks represent significant differences in cortisol at a particular time point between temperatures.

commonly found in their natural habitat (Bennett, 2005). Fish were held in a light-controlled room set to reflect the natural photoperiod (12 L: 12D) and were fed dry feed (BioVita starter (50% of #0 crumble and 50% #1 crumble)) at approximately 2% of their body weight per day. To monitor water quality of sub tanks, temperature (°C), dissolved oxygen (mg/L), and salinity (ppt) were measured daily using a hand-held YSI 556 MPS meter (YSI Inc., Yellow Springs, OH). Ammonia, nitrite, and nitrate concentrations, and pH were measured biweekly. pH was measured using a pinpoint pH monitor (American Marine Inc., Ridgefield, CT) or with commercial pH strips. A marine care multi test kit was used for ammonia, nitrite, and nitrate measurements (Red Sea, Houston, TX), and ammonia was additionally measured using a Hach pocket colorimeter (Hach Company, Loveland, CO). Mortality was checked daily, and dead fish were removed immediately to ensure water quality was not compromised. All handling, care, and experimental procedures used were reviewed and approved by the UC Davis Institutional Animal Care and Use Committee (IACUC Protocol 16591).

2.2. Temperature treatments

Following the two-week acclimation period, Delta Smelt were held at a control temperature of 17 °C and a high temperature of 21 °C for a week prior to sampling. Temperatures were gradually increased from 16 °C at a rate of 0.5–1.5 °C per day until desired temperatures were reached. These temperatures were chosen to reflect conditions Delta Smelt are expected to experience in their natural habitat. Delta Smelt are generally found in a temperature range of 15–20 °C in nature with <10% of Delta Smelt caught in water temperatures >20 °C (Bennett, 2005). In addition to daily temperature measurements, each tank had a designated HOBO temperature logger (Onset), which was exchanged between treatment sub tanks every two days and recorded temperatures (°C) every 15 min. All pre-experiment acclimation and temperature acclimation data are presented in Supplemental Table 1.

2.3. Experiment 1- Basal and maximal stress response to netting stress

The first goal of this study was to test basal and peak cortisol levels prior to and following a sublethal netting stress to provide a time course of cortisol induction in Delta Smelt. These trials were conducted after two weeks of acclimation to pre-experiment conditions and one week acclimation at 17 °C and 21 °C. First, three fish from each of the 12 replicates (four replicate sub tanks for each of the three post stress time points) were sampled for basal cortisol levels, resulting in 36 basal samples in each temperature treatment. Fish were netted from individual sub tanks, immediately euthanized with an overdose of tricaine methanesulfonate (500 mg/L MS-222; Finguel) buffered to a neutral pH

with sodium bicarbonate, weighed (g), measured for fork length (mm), and snap frozen in liquid nitrogen. This protocol took <3 min to ensure cortisol levels were not affected by handling stress. Care was taken to catch fish as efficiently and quietly as possible to minimize any disturbance to surrounding sub tanks.

Upon completion of basal cortisol sampling, remaining juvenile Delta Smelt were subjected to a sublethal 5-min netting stress, simulated by lifting the mesh baskets out of water from individual sub tanks so that only 1.5 cm of water separated the bottom of the net from aerial exposure. For the last 30 s of the 5-min netting stress, the basket was lifted further out of the water resulting in a 0.5 cm water depth. Although it was possible to stay fully submerged during the 5-min netting stress, most fish displayed stressed swimming behaviors and significant splashing, resulting in partial aerial exposure during these trials. A pretrial was conducted to ensure the netting stress did not result in significant mortality. Once the 5-min netting stress was completed, baskets were gently dropped back down to the bottom of sub tanks and lids were returned on top.

Three fish from each replicate were then sampled 15, 30, and 60 min following the termination of the netting stress, resulting in 12 samples per time point. All fish from each sub tank were sampled at the same time by lifting the basket and immediately placing fish into a bucket of MS-222. Netting stress trials were conducted on three sub tanks at a time in random order such that all sub tanks in a 400-L holding tank were sampled at once. This procedure minimized disturbance of sub tanks in other 400-L holding tanks (Supp. Fig. 1). Fish in the 17 °C treatment group were all sampled for basal and maximal cortisol on the same day. Identical methods were repeated on the following day to sample fish in the 21 °C treatment. Fish were fasted for 24 h prior to sampling. All samples were stored at –80 °C for later analysis.

Although care was taken to eliminate variables that likely influence cortisol in Delta Smelt (e.g., handling, stress, and metabolism), we acknowledge that true basal cortisol values were impossible to achieve due to the inability to eliminate all external stimuli, such as photoperiod and conspecifics (Barton and Iwama, 1991). Similarly, the need to balance stressor severity with reduced fish mortality may have limited our ability to achieve true maximal cortisol. However, we are confident that our range in basal to maximal cortisol values are ecologically relevant and provide critical insight into the stress response of this species.

2.4. Experiment 2- Predator cue

The second aim of this study was to investigate the effects of an environmentally relevant predator cue on the stress response of juvenile Delta Smelt. This trial was conducted after two weeks of acclimation to pre-experiment conditions and three weeks of acclimation at 17 °C.

Predator cues were prepared in advance of exposure trials by holding a largemouth bass (*Micropterus salmoides*) in an insulated and aerated cooler with 50 mL of water per g of bass for 24 h (Davis et al., 2019). Predator cue water was taken directly from coolers and stored in 60 mL syringes until introduction into sub tanks. To avoid additional disturbances, predator cues were injected through airline tubing via a two-outlet syringe coupler from outside holding tanks with closed lids. Airline tubing was affixed 1 cm below water level and around 3 cm downstream of the outflow valve, to increase cue distribution in sub tanks.

To introduce predator cues, three 60 mL syringes were pre-prepared so they could be added in sequential order. The first syringe had 60 mL tank water used to prime the tubing, followed by 60 mL of the predator cue and finalized with another 60 mL of tank water to avoid air bubbles and ensure all the predator cue had been effectively pushed through tubing. Two sub tanks per holding tank received the predator cue described above, while the control sub tank received three syringes filled with 60 mL tank water to act as a control treatment. The control fish were sampled 20 min following the injection of the tank water cue. The other two sub tanks, injected with predator cues, were sampled at 15 and 60 min to reflect the range of time points used in the basal and maximal cortisol trials in Experiment 1 (Supp. Fig. 1).

Fish were sampled one sub tank at a time by pulling out the basket and immediately placing fish into a bucket of MS-222, as described above for basal and maximal cortisol sampling. Fish were measured for fork length, weighed, and snap frozen in liquid nitrogen and stored at -80°C for later analysis. Fork length (mm) and wet weight (g) data for juvenile Delta Smelt measured on sampling days are presented in Supplemental Table 2. Six to eight fish from each replicate were analyzed from all three treatments (control, 15, and 60 min after predator cue), resulting in 28–32 samples per treatment.

2.5. Biochemical analysis of Delta Smelt stress response

2.5.1. Whole-body homogenization

The size of juvenile Delta Smelt in this study necessitated whole-body rather than plasma measurements so that enough sample was available to measure cortisol, glucose, and lactate in the same fish. Due to the complex dynamics of the HPI axis and resulting variability in cortisol levels, the head of each frozen fish was cut off with a sterile razor blade and the remaining tissue was ground to a fine powder, using a mortar and pestle over liquid nitrogen. Prior to homogenization, whole-body fish powder was weighed and kept on dry ice. Each sample was then homogenized in 4 mL ice-cold $1\times$ phosphate-buffered saline (PBS buffer: 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium phosphate and 1.8 mM monopotassium phosphate, and pH 7.4) with the addition of protease inhibitors (Roche Molecular Systems, Inc), using a hand-held homogenizer (PRO Scientific, Oxford, CT). Samples were then split into 4 equal parts for whole-body cortisol, glucose, lactate, and protein analyses. Homogenate (1 mL) for cortisol analysis was added to a 9 mL Pyrex glass tube for same day extraction. The homogenates designated for glucose, lactate and protein analyses were centrifuged for 30 min at 14,500g at 4°C . The supernatant was then extracted and stored at -80°C for later analysis in 1.5 mL microcentrifuge tubes.

2.5.2. Cortisol extraction and analysis

Cortisol extraction followed methods outlined in Hasenbein et al., 2013. Volumes of solutions were optimized for use in juvenile Delta Smelt in this study. Each cortisol homogenate sample was spiked with 2.5 mL of diethyl ether, vortexed for 1 min and then centrifuged for 7 min at 3200g at 4°C . The supernatant was extracted carefully with a glass pipette and transferred to a new 9 mL Pyrex glass tube. To ensure maximal extraction, this process was repeated two times per sample and extracted supernatant from all three washes were combined (Cachat et al., 2010; Hasenbein et al., 2013). Samples were left in the fume hood overnight for complete diethyl ether evaporation. After evaporation,

samples were resuspended in either 200 or 500 μL 1xPBS (depending on weight of fish), vortexed, and stored at -80°C until later analysis.

Whole-body cortisol levels were measured using an enzyme immunoassay (EIA) kit, following the manufacturer's instructions (Salivary Cortisol Immunoassay, Salimetrics LLC). Samples were run in duplicate, and cortisol concentrations ($\mu\text{g dL}^{-1}$) were calculated using a four-parameter sigmoid standard curve. Protein concentrations of each homogenate were measured using the bicinchoninic acid method (Pierce, Thermo Fisher Scientific Inc.). Samples were run in duplicate, and protein concentration ($\mu\text{g mL}^{-1}$) was calculated using a linear standard curve. Samples outside the range of standard curve values were eliminated from analyses. Cortisol concentrations were corrected for dilution and normalized to both weight (ng cortisol per g fish) and protein content (pg cortisol per μg protein). Cortisol values normalized to fish weight were used in statistical analyses.

2.5.3. Glucose and lactate analyses

Glucose and lactate homogenate samples were thawed on ice. Glucose (glucose assay kit, Sigma-Aldrich), and lactate (lactate assay kit II, Sigma-Aldrich) were performed using commercial test kits following the manufacturer's instructions. Samples were run in duplicate and glucose and lactate concentrations (ng mL^{-1}) were calculated using a linear standard curve. Samples outside the range of standard curve values were eliminated from analyses. Glucose and lactate values were normalized to fish weight (μg glucose/lactate per g fish).

Finally, protein to weight ratios were calculated by dividing total protein ($\mu\text{g mL}^{-1}$) by weight of fish (g).

Standard curves of all assays had r^2 values over 0.98.

2.6. Statistical analyses

Statistical analyses were performed using R version 4.1.2 (R Core Team, 2021). To investigate the stress response in juvenile Delta Smelt following a sublethal netting stress, two-way nested ANOVAs followed by Tukey's *post-hoc* tests were employed using time and temperature as factors on whole-body cortisol, glucose, and lactate concentrations, nested by replicate sub tanks. To test the influence of a predator cue, one-way nested ANOVAs followed by Tukey's *post-hoc* tests were performed on whole-body cortisol, glucose, and lactate concentrations, tested by treatment and nested by replicate sub tanks. Differences in protein to weight ratios between all trials were determined using a Kruskal-Wallis test followed by pairwise Wilcoxon signed rank tests with Bonferroni correction. To test if temperature acclimation influenced mortality in Experiment 1, a Mann-Whitney *U* test was performed. Data were tested for normality and heteroscedasticity visually by plotting Q-Q, density and residual plots of the linear model and statistically using the Shapiro-Wilk and Levene's test. When necessary, data were transformed using the *bestNormalize* package to meet assumptions (Peterson, 2017). Data are presented as means $\pm$ standard error of the mean (SEM) and differences between means were deemed significant at $p < 0.05$.

3. Results

3.1. Cortisol response

3.1.1. Experiment 1- Basal and maximal stress response after netting stress

To investigate the kinetics of the generalized stress response in juvenile Delta Smelt, basal and maximal whole-body cortisol prior to (basal) and following a significant and sublethal netting stress were tested at three distinct time points (15, 30, and 60 min) at both 17 and 21°C . There was a significant effect of netting stress on cortisol concentrations at all time points following the stressor at both temperatures (Fig. 1; nested ANOVA: $F_{3,134} = 84.76$; $p < 0.0001$). There were no significant differences in cortisol levels from 15 to 60 min at either temperature. Cortisol levels started to decrease after 15 min at 17°C (15–60 min; $p = 0.14$) and after 30 min at 21°C (30–60 min; $p = 0.58$).

However, as decreases were non-significant, we assume peak cortisol is present within 15 min and recovery of cortisol takes longer than one hour at both 17 and 21 °C. There was a significant effect of acclimation temperature on the stress response in Delta Smelt, where cortisol was over twice as high 30 and 60 min following a netting stress at higher temperatures (nested ANOVA: $F_{4,134} = 7.47$; $p < 0.0001$).

3.1.2. Experiment 2- Predator cue

Delta Smelt displayed a significant increase in whole-body cortisol following a largemouth bass predator cue (Fig. 2; nested ANOVA: $F_{2,72} = 22.82$; $p < 0.0001$). There was a slight and non-significant decrease in cortisol from 15 to 60 min ($p = 0.33$) suggesting peak cortisol was reached by 15 min and recovery had not occurred by one hour. The predator cue was likely perceived as a substantial stress due to the 4-fold increase in cortisol ($p < 0.0001$); approximately half the increase induced by the severe netting stress we performed aimed to measure maximal cortisol in this species.

3.2. Glucose and lactate

3.2.1. Experiment 1- Basal and maximal stress response after netting stress

There was no evidence of the secondary stress response in juvenile Delta Smelt, as whole-body glucose (nested ANOVA: $F_{3,124} = 0.04$; $p = 0.99$) and lactate (nested ANOVA: $F_{3,127} = 1.01$; $p = 0.39$) concentrations remained similar to basal levels following the netting stress at both 17 and 21 °C (Fig. 3).

Temperature had a significant effect on whole-body glucose (nested ANOVA: $F_{4,124} = 8.09$; $p < 0.0001$) and lactate (nested ANOVA: $F_{4,127} = 4.23$; $p < 0.01$) concentrations, where Delta Smelt held at 17 °C displayed higher levels in metabolites than those held at 21 °C. Delta Smelt held at 17 °C had significantly greater glucose concentrations 30 and 60 min following the netting stress, and significantly greater lactate concentrations in the control group only ($p < 0.05$) (Fig. 3).

3.2.2. Experiment 2- Predator cue

Whole-body lactate concentrations were unaffected by the predator cue (nested ANOVA: $F_{2,70} = 0.62$; $p = 0.54$), whereas whole-body glucose concentrations were significantly decreased at 60 min following the predator cue (nested ANOVA: $F_{2,71} = 6.54$; $p < 0.01$) (Fig. 4).

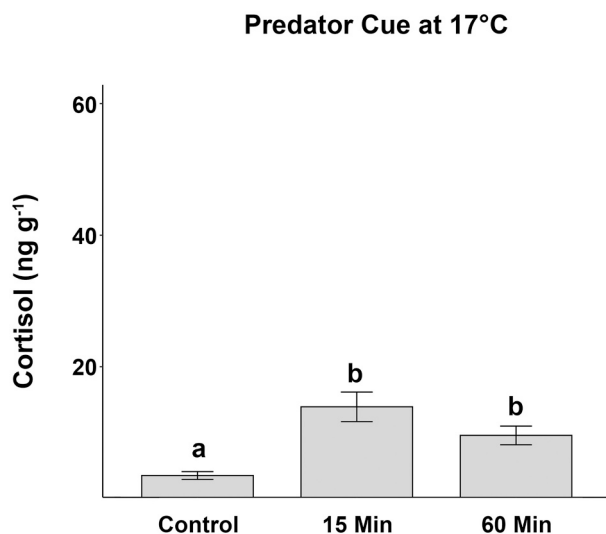


Fig. 2. Whole-body cortisol measurements (ng g⁻¹) in juvenile Delta Smelt prior to and following (15 and 60 min) exposure to a largemouth bass predator cue at 17 °C ($n = 27$ – 29). Data are presented as mean $\pm$ SEM and different letters represent significant differences in cortisol between sampling times.

3.3. Protein to weight ratios

Delta Smelt raised at 21 °C had significantly lower protein to weight ratios compared to fish raised at 17 °C (Supp. Fig. 2; Kruskal-Wallis: $\chi^2 = 33.16$, $df = 2$, $p < 0.0001$).

3.4. Mortality

In Experiment 1, there was no significant difference in mortality between temperature treatments (Mann-Whitney U: $U = 46$, $p = 0.103$), suggesting that the timing of temperature exposure induced sublethal effects only. There was no mortality associated with the netting stress.

4. Discussion

The ability to perceive and respond to environmental stressors will ultimately affect an organism's capacity to tolerate environmental change and survival in their natural habitat. After exposure to acute stressors, measured changes in cortisol are commonly used as indicators of the primary stress response in fish, while subsequent changes in glucose and lactate concentrations are used as markers of the secondary stress response (Barton, 2002). In the present study, we investigated the primary and secondary stress response in juvenile Delta Smelt following a netting stress at two ecologically relevant temperatures (17 and 21 °C). The stress response was assessed through whole-body cortisol, glucose, and lactate measurements at 15, 30, and 60 min following stressor exposure. Overall, we found that Delta Smelt mounted a significant primary stress response with increased cortisol relative to basal levels, which was exacerbated at higher temperatures (Fig. 1). Our results also suggest that Delta Smelt perceive predator cues as significant stressors due to the elevation of their cortisol response (Fig. 2). The secondary stress response was not detected as glucose and lactate concentrations did not increase following stressor exposure in either experiment (Figs. 3 & 4). However, decreased secondary stress response measures and protein to weight ratios between temperature trials suggest that chronic elevated temperature may compromise their stress response, as Delta Smelt held at 21 °C had reduced available energy in the form of free glucose, lactate, and total protein compared to those held at 17 °C (Fig. 3; Supp. Fig. 2).

4.1. Cortisol response

4.1.1. Experiment 1- Basal and maximal stress response after netting stress

The time course of cortisol induction varies significantly between species of fish and has been related to lifestyle differences, such that sedentary species tend to take longer to display increased cortisol (Vijayan and Moon, 1994; Wright et al., 2007). Initial increases in cortisol resulting from exposure to stressors have been found to range from 2.5 min in striped bass (*Morone saxatilis*) to 2 h in the sea raven (*Hemitripterus americanus*), with a mean response latency of 12.5 min (Pankhurst, 2011; Tomasso et al., 1996; Vijayan and Moon, 1994). Delta Smelt displayed similar timing in onset of cortisol induction with significant increases at 15 min when fish were held at both 17 and 21 °C; however, as 15 min was the earliest time point examined, initial increases may have begun any time after stressor exposure up to 15 min (Fig. 1).

In most species of teleost fish, it takes 0.5–1 h to reach peak cortisol levels following a stressor, although exceptions exist (reviewed in: Barton, 2002). As Delta Smelt are a highly migratory species, it is not surprising that they displayed peak cortisol values at 15 min and earlier than the average teleost species (Sommer et al., 2011). Recovery of cortisol to basal levels has been shown to range from 2 to 6 h up to 24–48 h in teleosts (Barnett and Pankhurst, 1998; Pickering et al., 1982; Robertson et al., 1988; Vijayan and Moon, 1994). Although Delta Smelt started to display decreases in cortisol at both temperatures, cortisol values at all timepoints were significantly greater than basal levels,

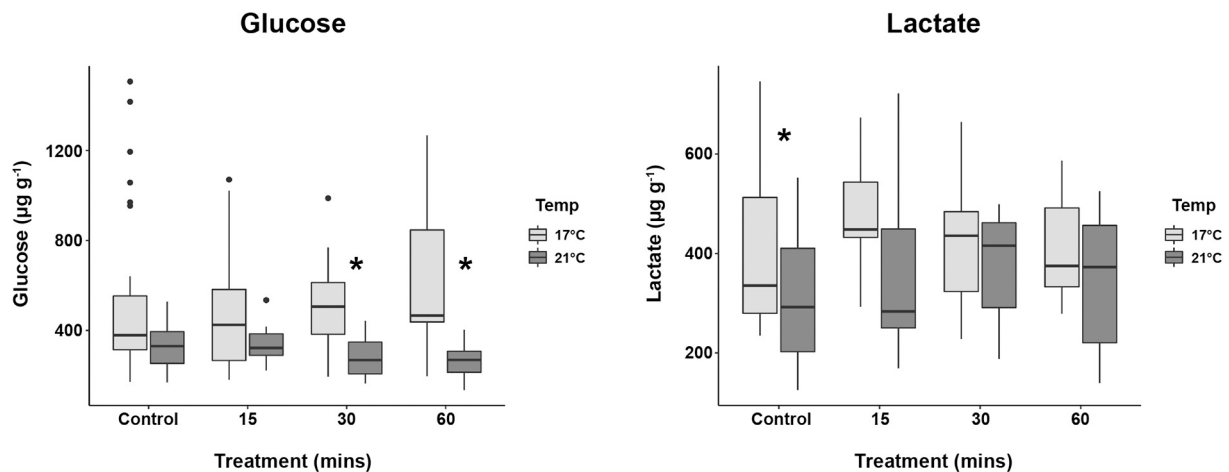


Fig. 3. Whole-body glucose ($n = 10\text{--}32$) and lactate ($n = 9\text{--}34$) measurements ($\mu\text{g g}^{-1}$) in juvenile Delta Smelt prior to and following a netting stress at 17 °C (lighter boxes) and 21 °C (darker boxes). The line of the boxplot represents the median of the data. The box represents the inter-quartile range (IQR), the whiskers extend 1.5 x IQR, and the black points represent values outside 1.5 the IQR. Asterisks represent significant differences in glucose or lactate at a particular time point between temperatures.

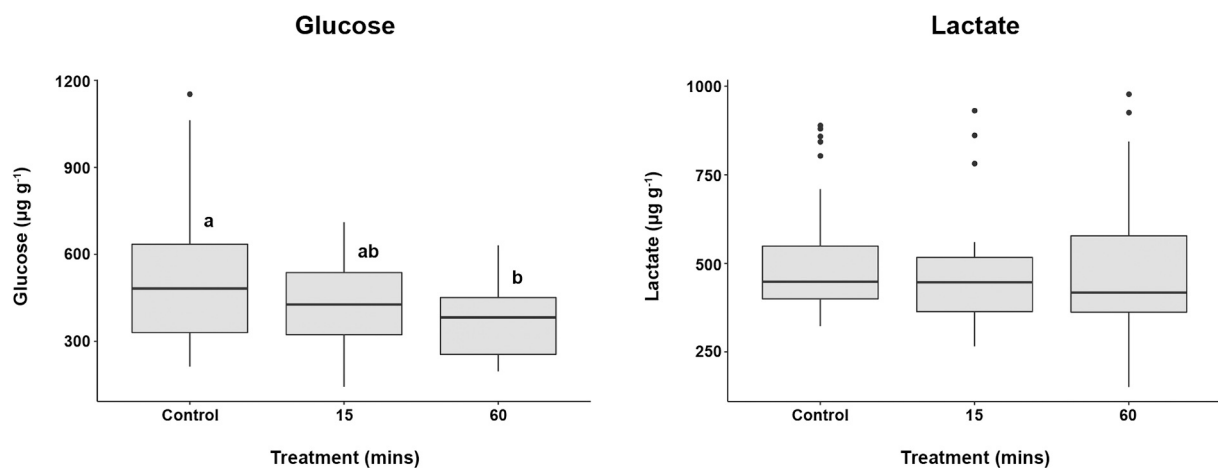


Fig. 4. Whole-body glucose ($n = 25\text{--}29$) and lactate ($n = 26\text{--}29$) measurements ($\mu\text{g g}^{-1}$) in juvenile Delta Smelt prior to and following a largemouth bass predator cue stress at 17 °C. The box represents the inter-quartile range (IQR), the whiskers extend 1.5 x IQR, and the black points represent values outside 1.5 the IQR. Letters represent significant differences in metabolite concentrations between sampling times.

suggesting recovery of cortisol was not reached in this current study. Extension of time points should be included in future studies with the aim of identifying full recovery time in juvenile Delta Smelt. This will not only provide important information to our overall understanding of cortisol dynamics in this species, but it will also be beneficial when planning the Delta Smelt population supplementation process (USFWS, 2019; 2020) because knowledge of the duration of time needed for cortisol to return to basal levels can be used in setting limits on release time following handling stress (Ashley, 2007; Finstad et al., 2003; Iversen et al., 1998; Schreck et al., 1989).

There is also considerable variability in the magnitude of the cortisol response between different species of teleost fish, with peak values in plasma cortisol ranging from <15 ng/mL in Atlantic cod (*Gadus morhua*) to nearly 2000 ng/mL in striped bass (*Morone saxatilis*) (reviewed in: Barton, 2002; Hemre et al., 1991; Mazik et al., 1991). Temperature had a significant effect on the magnitude of whole-body cortisol in juvenile Delta Smelt in the current study, where levels were over twice as high at 21 °C compared to 17 °C at both 30 (17 °C: 20.3 ± 3.1 ; 21 °C: 48.4 ± 8 ng g^{-1} body weight) and 60 (17 °C: 11 ± 1.4 ; 21 °C: 33.5 ± 7.7 ng g^{-1} body weight) min following the netting stress (Fig. 1). This data suggests that temperature acted as an additional stressor to Delta Smelt and is

thus a variable that should be considered for future supplementation and release.

When whole-body cortisol levels were assessed in adult zebrafish (*Danio rerio*) following a 1-min air exposure stressor, fish displayed a 7-fold increase in cortisol levels compared to unstressed control fish, 20 min following the stressor (17.5 ± 5.4 ng g^{-1} body weight; Alderman and Vijayan, 2012). These results are comparable to those obtained for Delta Smelt in the current study that displayed an approx. 8-fold increase in whole-body cortisol 15 min after exposure to a 5-min netting stress with partial aerial exposure (Fig. 1; 17 °C: 25 ± 2.9 ; 21 °C: 36.3 ± 3.9 ng g^{-1} body weight; $p < 0.0001$). Our Delta Smelt cortisol levels were in the same range as those previously reported for this species. Supplemental Table 3 includes whole-body cortisol measurements corrected by total protein ($\text{pg } \mu\text{g}^{-1}$) for direct comparison to other studies displaying cortisol in a similar manner, specifically those examining whole-body cortisol in late-larval and juvenile Delta Smelt (Hasenbein et al., 2013, 2016a, 2016b).

4.1.2. Experiment 2- Predator cue

Predation stress from visual, acoustic, and olfactory cues is found to induce the primary stress response and increase cortisol in different

species of fish (Barcellos et al., 2007; Barkhymer et al., 2019; Barreto et al., 2014; Remage-Healey et al., 2006). Delta Smelt displayed a significant increase in whole-body cortisol following a largemouth bass predator cue. Similarly to Experiment 1, cortisol peaked by 15 min and due to non-significant decreases at 60 min, recovery of cortisol was not reached with the current time course. The predator cue elicited a 4-fold increase in cortisol levels, suggesting it was perceived as a significant stressor to Delta Smelt (Fig. 2). Plasma cortisol levels doubled in frillfin goby (*Bathygobius soporator*) and Nile tilapia (*Oreochromis niloticus*) following exposure to conspecific chemical alarm cues (Barreto et al., 2014; Sanches et al., 2015). Whole-body cortisol in zebrafish doubled in the presence of a predator and increased by one third following visual contact with that same predator (Barcellos et al., 2007). At the higher range, whole-body cortisol in zebrafish displayed a 7 and 8-fold increase upon exposure to the odor of dead conspecifics and chemical alarm cues, respectively (Barkhymer et al., 2019; Oliveira et al., 2014). In some cases, antipredator behaviors are induced without the activation of the HPI axis. After exposure to a predator odor, Nile tilapia displayed antipredator responses, such as decreased locomotion and increased ventilation rate with no corresponding changes in plasma cortisol or glucose (Miyai et al., 2016).

In this study, predator cue trials were only conducted at 17 °C; however, based on the results of the netting stress at 17 and 21 °C, we would predict that the predation response in Delta Smelt would be exacerbated at higher temperatures. Davis et al. (2019) explored the effects of temperature on predation and anti-predator responses in juvenile Delta Smelt after exposure to conspecific and predator alarm cues. Fish held at 21 °C were preyed upon more often and displayed intensified swimming behaviors associated with stress, with a longer time to recovery to baseline behaviors, compared to those held at 17 °C or cycling temperatures (17–21 °C) (Davis et al., 2019). These results combined with results from the current study suggest that temperature has a significant effect on the stress response in Delta Smelt by intensifying anti-predator behaviors and increasing cortisol induction. As the stress response is energetically expensive, prolonged periods at increased temperatures will likely have long-term effects on the energetic reserves and overall fitness of Delta Smelt (Barton and Iwama, 1991; Schreck, 2010; Sokolova et al., 2012; Tort, 2011).

4.2. Glucose and lactate

4.2.1. Experiment 1- Basal and maximal stress response after netting stress

There were no differences in whole-body glucose and lactate measurements prior to and following a sublethal netting stress, despite significant increases in cortisol (Fig. 3). Although we expect an increase in glucose concentration following elevated cortisol, there is much inconsistency in the literature regarding the effects of cortisol on carbohydrate metabolism in fishes. Reliable data on plasma glucose and liver glycogen is challenging to obtain, partially due to substantial within species variability (Mommensen et al., 1999). There are many other examples in fishes where glucose and lactate remain unchanged following significant increases in cortisol (Andersen et al., 1991; Cortés et al., 2018; Herrera et al., 2016; Rotllant and Tort, 1997). Following a 3-min handling stress, juvenile rainbow trout (*Oncorhynchus mykiss*) exhibited initial increase in both plasma cortisol and lactate concentrations by 1-h post-stress, whereas plasma glucose displayed a more gradual increase with higher concentrations at 3- and 6-h post-stress (Vijayan et al., 1994). One possible explanation for the lack of any observable changes in metabolite concentrations following stressor exposure is that our sampling period was not long enough to encompass the rise in glucose or lactate concentrations. The negligible effect on lactate concentration may alternatively suggest that the netting stress did not cause an exhaustion of energetic reserves and a shift to anaerobic respiration (Pankhurst, 2011). Lastly, since the size of juvenile Delta Smelt necessitated whole-body glucose and lactate measurements, another possible explanation is that changes in plasma levels could not be detected with a

whole-body approach.

The metabolites glucose and lactate were greater in fish held at 17 °C compared to those held at 21 °C, suggesting that one week at higher temperatures was sufficient to cause a significant difference in available energy in Delta Smelt. Lactate is a known substrate for increasing glucose concentrations and, thus, may explain the correlation of decreased glucose and lactate concentrations in Delta Smelt held at higher temperatures (Polakof et al., 2012; Suarez and Mommensen, 1987). Chase et al. (2016) investigated the effect of low food availability on the physiological condition and stress response of tidewater goby (*Eucyclogobius newberryi*). Comparable to results of the current study, they found that higher glucose and lactate concentrations were correlated with fish in better condition, while cortisol had a negative correlation with fish condition (Chase et al., 2016).

In addition to displaying both reduced glucose and lactate concentrations at higher temperatures, Smelt raised at 21 °C had significantly lower protein to weight ratios compared to fish raised at 17 °C. As protein is another important energy source utilized by fish, this combined data suggests higher temperatures may increase energy depletion in juvenile Delta Smelt.

4.2.2. Experiment 2- Predator cue

Similar to the netting stress, the predator cue stress did not cause any significant changes in whole-body lactate; however, there was an interesting and unexpected finding in which whole-body glucose concentrations were significantly decreased at 60 min compared to controls, with intermediate values at 15 min (Fig. 4). Although most species of fish display elevated plasma glucose following increased cortisol, significant decreases in plasma glucose have also been reported (Foster and Moon, 1986; Van Der Boon et al., 1991; Vijayan et al., 1991). As we investigated changes in whole-body glucose rather than plasma glucose alone, a likely explanation is that our results reflect the complex dynamics of gluconeogenesis and glycolysis given that glucose levels are a result of concentrations in specific organs as well as in circulation. Our results may highlight metabolism of available glucose prior to its increased production as a secondary response of stress and cortisol increase, resulting in an overall reduction of whole-body glucose. Future experiments extending time points to further explore secondary stress response variables will shed light on this finding.

4.3. Concluding remarks

This study is the first to characterize cortisol dynamics and kinetics and secondary physiological changes associated with stress in Delta Smelt. A better understanding of the stress response in Delta Smelt permits the use of cortisol as a biomarker of stress in experiments aimed to characterize stressors in their natural environment. As such, we show that Delta Smelt perceive an ecologically relevant predator cue as a significant stressor based on cortisol increase relative to basal levels. Results of our study have important implications for the consideration of aquaculture and supplementation in endangered Delta Smelt. Chronic or repeated stress may limit the bioenergetic capacity in fish by decreasing energetic reserves (Wendelaar Bonga, 1997). Energy required to maintain homeostasis is greater during stress; therefore, it is likely that fish under chronic stress will experience a reduction in energy available to allocate towards growth, fecundity, and development, which will likely have long-term effects on their survival and fitness (Sokolova et al., 2012). Since it is known that tolerance to stress is limited in Delta Smelt, care should be taken when setting optimal holding conditions in both aquaculture settings as well as before and during release for future supplementation (Davis et al., 2019; Jeffries et al., 2016, 2018; Komoroske et al., 2014, 2015; Swanson et al., 1996, 2000). As transport and handling stress during supplementation is likely very high, it will be vital to prioritize conditions that reduce stress and optimize available energy in this sensitive species (Lessard et al., 2018; Swanson et al., 1996). Our results suggest it would be advantageous to release fish in an

environment with moderate temperatures and reduced predation to minimize stress and maximize Delta Smelt survival.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the hatchery staff and volunteers at UC Davis Fish Conservation and Culture Lab for maintaining the captive population of Delta Smelt used in this research. We also thank Jordan Colby, Sarah Baird, and Alexis Lundquist for their help with fish care, experimental set-up, and sampling. This research was made possible by funding from the USFWS # F19AC00943 to AET, REC, T-CH and NAF, and the University of California, Davis Agricultural Experiment Station (CA-D-ASC-2252-H to AET and CA-D-ASC-2098-H to NAF). The findings and conclusions in this article are those of the authors and do not necessarily represent the view of the U.S. Fish and Wildlife Service. USBOR, R20AC00027 funding to T-CH was used for Delta Smelt rearing and maintenance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbpa.2022.111303>.

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