EVALUATING THE EFFECTS OF ELEVATED WATER CONDUCTIVITY ON THE SOUTHERN REDBELLY DACE (*CHROSOMUS ERYTHROGASTER)* AT DIFFERENT LIFE HISTORY STAGES

By

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A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science in Biology

Austin Peay State University
2020

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Austin Hannah 07/31/2020

ACKNOWLEDGEMENTS

I would like to first thank my supervisor, Dr. Mollie Cashner, for her continual support and much-needed guidance throughout the course of my graduate school experience. Thank you for facilitating my transition back into school and helping me develop the tools necessary to be successful scientist; skills that I will carry with me for the rest of my career. I would like to thank Dr. Leslie Hiatt for her help answering my (many, many) questions involving analytical and instrumental chemistry. I would like to thank Erin Schwarzbach for all her help in the field and lab and for being an outlet for the many frustrations encountered throughout my project. I would like to thank Austin Peay State University Co-STEM as well as North American Native Fish Association for their financial assistance; without which my project would have not been possible. I want to thank my family for their support and for kindling within me a passion for all living things that helped sustain my focus throughout this project. Lastly, I'd like to thank my wife, Michelle, for being a solid foundation, for offering constant reassurance, and for having incomparable patience over the last two years.

ABSTRACT

The Blackside Dace (*Chrosomus cumberlandensis*; BSD) is a federally threatened minnow that has been extirpated from 31 streams in Tennessee and Kentucky since 1978. Although there is strong evidence linking elevated conductivity levels and low BSD occurrence, the mechanistic relationship between the two variables is poorly understood. The goal of this project is to further elucidate proximate explanations for the progressive extirpation and contemporary genetic disjunction of BSD populations using a closely related species, *Chrosomus erythrogaster (*Southern Redbelly Dace, SRBD). We hypothesize that elevated water conductivity affects reproduction of SRBD and causes sub-lethal effects which contribute to reduced fitness in adults. First, we reconstituted water chemically analogous to streams within the historic range of BSD reflecting varying levels of mining related impairment. Using reconstituted water, we subsequently performed five separate experiments on SRBD at different life stages testing both acute and chronic stress responses to elevated water conductivity. We found a significant negative relationship between water conductivity level and proportion of SRBD egg hatch (GLM; p <0.001, n = 600 eggs from 7 individuals). Our acute, sub-lethal test results support the hypothesis that elevated conductivity elicits an acute stress response from adult SRBD exhibited by detection of significantly elevated waterborne cortisol (ANOVA; $F_{2,9} = 7.34$, $p = 0.013$, n = 12) as well as increased oxygen consumption (ANOVA; $F_{2,17} = 11.5$, p <0.001, $n = 27$). Furthermore, evidence of chronic stress to adult SRBD was detected after prolonged exposure to high conductivity; individuals in impaired treatments experienced reduced growth over a five week period compared to controls (ANOVA*;* $F_{1,18} = 7.11$, $p = 0.016$, 95% CI: -0.27, -0.04) and, although not significant, we detected a trend indicating hypothalamic-pituitary-interrenal axis disruption reflected by reduced cortisol response in high conductivity groups (Control: 6.96 ng g-

1 60 min-1, *SD* = 4.6; High conductivity: 4.03 ng g-1 60 min-1, *SD* = 2.28; T-test; $p = 0.16$, $n = 12$). Our results provide the first evidence demonstrating the potential negative fitness costs associated with elevated water conductivity related to surface mining on a representative fish species at multiple life-history stages.

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I. Introduction

The Appalachian Blue-Ridge forest ecoregion encompasses approximately 62,000 square miles and extends south from New York state to northern Alabama in a 1,500-mile unbroken chain. This region contains an abnormally high number of relic and endemic species due to its long-term geologic stability (Bernhardt and Palmer 2011). Central Appalachia harbors the most biodiverse freshwater systems in North America and contains roughly 10% of the global amphibian and mussel diversity (Stein et al. 2000). Headwater streams represent a disproportionate percentage of river networks in Central Appalachia (USEPA 2005) and play a critical role in maintaining the biodiversity of freshwater systems. These small catchments offer a diversity of habitats that act as a refuge for sensitive flora and fauna from predators, competition, and invasive species (Meyer et. al 2007). Although fish biodiversity is relatively low in Appalachian headwater streams, inhabitants possess unique physio-chemical requirements (USEPA 2005). Headwater streams are especially vulnerable to small scale biotic and abiotic changes as a consequence of their small size and, as a result, headwater species are most likely to be affected by anthropogenic changes (USFWS 2017).

Surface mining impacts

In Central Appalachia, surface mining for coal is arguably the most influential factor contributing to headwater stream alteration and aquatic ecosystem degradation (Bernhardt and Palmer 2011, USEPA 2011a, Lindberg et al. 2011). Central Appalachia supplies roughly 18% of the nation's coal (Bernhardt and Palmer 2011) and The United States Environmental Protection Agency (USEPA) predicted that by 2012, surface mining for coal impacted about 7% of the 4.86 million hectares of forested land within the Appalachian coalfield region of West Virginia,

Kentucky, and Tennessee (USEPA 2011a). Over 2,000 miles of headwater streams have been covered by valley fill activities in the last forty years (USEPA 2011a). In addition to effectively eliminating streams, surface mining has a number of well-documented adverse effects on aquatic and terrestrial ecosystems including increased sedimentation, heavy metal pollution, acidification, and increased specific conductivity (USFWS 2011a; Bernhardt and Palmer 2010; Jiminez et al. 2009; Lindbergh et al. 2000).

Elevated conductivity

The specific conductivity of water is a measure of its ability to conduct an electric current, therefore it is a direct measurement of the ionic strength of a salt solution (measured as μS/cm, USEPA 2011). Appalachian streams unaffected by surface mining typically range between 30-110 μS/cm (USEPA 2011b); however, severely impaired streams have up to thirty times the background conductivity levels (Pond et al. 2008; Palmer et al. 2010; Lindberg et al. 2011). Regardless of reclamation strategy, ionic concentrations downstream of inactive mining operations persist for decades after operational cessation (Lindberg et al. 2011; Bernhardt and Palmer 2011). Surface mine discharge in Central Appalachia is uniquely characterized by the disproportionately high concentration of Ca^{2+}, Mg^{2+} , SO_4^{2-}, and CO_3^{2-} ions as a result of excess pyrite, calcite, and dolomite that comprise the majority of mining overburden within the region (USEPA 2011b). Hardness is typically elevated in mining-affected streams and pH is circumnuetral to alkaline as a result of excess bicarbonate salts leached into the watershed (Pond et al. 2008; Bryant et al. 2002).

Elevated specific conductivity (conductivity; measured in μS/cm) caused by surface mining is undoubtedly one of the most appreciable stressors for freshwater organisms in Central Appalachia (Bernhardt and Palmer 2011; USEPA 2011b). A number of field studies examining

elevated conductivity within mining affected streams of West Virginia, Kentucky, and Virginia have found significant decreases in family and genus-level richness of both macroinvertebrate and fish assemblages (Hitt and Chambers 2014; Hopkins et al. 2013; Timpano 2011; Bernhardt and Palmer 2011; Pond et al. 2008; Stauffer and Ferreri 2002). In Appalachia, macroinvertebrate richness and abundance are significantly altered at conductivity levels as low as 300 μS/cm, whereas fish community shifts have been observed between 600-1000 μS/cm (Hitt and Chambers 2014; Pond et al. 2008). In healthy Appalachian streams, mayfly nymphs comprise up to 50% of the macroinvertebrate biomass, and at high disturbance sites (>1000 μS/cm) this group is virtually eliminated, along with reductions in over 30% of caddisfly and stonefly larvae (Pond et al. 2008). Clements and Kotalik (2015) demonstrated macroinvertebrate community tolerance to elevated conductivity is correlated with the background conductivity of occupied streams. In the same region, communities within low conductivity streams (60-72 μS/cm) experience greater toxicity responses compared to those found within higher conductivity streams (200-250 μS/cm; Clements and Kotilak 2015). In 2011, the Environmental Protection Agency (EPA) issued a maximum conductivity benchmark of 300 μS/cm to protect 95% of aquatic life in ecosystems within Central Appalachian regions encompassing ecologically similar Ecoregions 68, 69, and 70 (USEPA 2011a).

Despite numerous studies clearly showing disturbed aquatic communities downstream of surface mining activity (Hitt and Chambers 2014; USEPA 2011a; Petty et al. 2010; Pond et al. 2008) there have been few, conflicting experimental investigations assessing the impact of alkaline surface mining pollution on freshwater organisms, and none published thus far on fish. The inconsistent results of previous toxicity experiments are, in part, due to the use of non-representative species that are comparatively tolerant to salinity fluctuations (Kunz et al. 2013;

USEPA 2012; Merricks et al. 2007). Kunz et al. (2013) performed toxicity experiments using both Appalachian representative and non-representative invertebrate taxa and found that native taxa experienced adverse effects to alkaline mine effluent at significantly lower concentrations than non-native taxa, illustrating the importance of species selection for laboratory testing in studies inferring toxicity of field-derived water quality measurements.

The biochemical mechanisms responsible for the adverse effects of elevated water conductivity on freshwater organisms are attributed to osmotic stress (Cormier et al. 2013; USEPA 2011a; USEPA 2011b). Both freshwater invertebrates and fish utilize semipermeable membranes within gill epithelia for critical osmoregulatory functions such as internal pH regulation, removal of metabolic waste, secretion of hormones, initiation of embryonic development, and generation of cellular energy (Evans 2009; Bradley 2009; Tarin et al. 2000). In stenohaline, freshwater fauna these activities are dependent on passive ionic transport processes driven by a concentration gradient between high internal and low external ionic concentrations, disruption of this ionic gradient results in a loss of ionic co-transport functionality (USEPA 2011a; Evans 2009; Bradley 2009). Increased concentrations of bicarbonate salts and sulfates in water can affect the uptake of Cl^- ions in gill lamina, preventing the removal of CO_2 and subsequently disrupting the internal balance of essential ions such as K^+, H^+, and NH_4 leading to physiological stress and reduced survivorship (USEPA 2011a, Avenet and Lingnon 1985).

In addition to direct mortality, the effects of acute osmoregulatory stress and prolonged, chronic stress on fishes contribute to reduced survivorship in wild populations (Barton 2002). In fish, acute physiological responses to environmental stressors manifest as primary and secondary phases (Helfman et al. 2009). Primary responses include activation of the hypothalamic-

pituitary-interrenal (HPI) axis and the release of cortisol into the bloodstream within minutes (Sadoul et al. 2019; Barton 2002). Genetic, developmental, and environmental factors can influence the corticosteroid stress response in fish, contributing to the wide variation in release rates reported for freshwater fish in experimental studies (Barton 2002). Additional acute, sub-lethal effects induced by whole-body stress manifest as secondary responses leading to increased oxygen consumption, behavioral modifications, and increased gill permeability (Sopinka et al. 2016; Echols et al. 2010; Merricks et al. 2007; Mount et al. 1997). Chronic exposure to an environmental stressor and persistent elevated cortisol leads to the activation of tertiary responses resulting in prolonged diversion of energy and subsequent reduced body condition, reduced growth, reduced reproductive output, and reduced immune function (Helfman et al. 2009; Barton 2002). Chronic stress in fish may also cause desensitization of the HPI axis, reflected by a reduced ability to mount a cortisol response (Barton 2002; Reid et al. 1998, Hontela 1997).

Surface mining and Blackside Dace

The adverse response of fish communities to elevated water conductivity is less studied and less straightforward than macroinvertebrate community responses; however, the existence of the Blackside Dace (*Chrosomus cumberlandensis;* BSD) is jeopardized as a direct result of coal mining activity within its home range (Yates 2017; Hitt et al. 2016; McAbee 2013; USFWS 2015). The BSD is a federally threatened minnow endemic to headwater streams characterized by low conductivity and low temperature (Hitt et al. 2016; Black et al. 2013). The current range of BSD extends across the upper Cumberland River drainage in TN and KY, one watershed in the upper Kentucky River, and the Powell and Clinch River watersheds in Virginia (Black et al 2013b, USFWS 2015). As of 2015, there were 77 active coal mining permits in the upper

Cumberland River drainage of Tennessee, and an additional 169 active coal mines in the upper Cumberland River watershed of Kentucky (USFWS 2015). The BSD has been extirpated from 31 streams since its description in 1978 (Etnier and Starnes), and the U.S. Fish and Wildlife Service (USFWS 2015) recovery plan for BSD states that anthropogenic factors, including elevated water conductivity as a result of surface mining activity, pose the greatest present threat to the species.

The first thorough population genetic analysis of BSD revealed a high degree of isolation among current populations caused by factors other than geographic distance; these findings are against a backdrop of mtDNA data indicating widespread historic connectivity (Cashner and Johansen, unpub data). Streams currently containing stable BSD populations are characterized by conductivities between 60-170 µS/cm (Jacob Culp, personal communication 2019) and field studies have documented BSD intolerance to streams above 340 µS/cm (Yates 2017; Hitt et al. 2016; Black and Mattingly 2007). These observations suggest that the observed BSD metapopulation disjunction is partially influenced by a hostile stream matrix fragmented by mining-impaired streams. Although there is strong evidence linking increased water conductivity to decreased BSD occurrence, the mechanistic relationship between the two variables is poorly understood (Black and Mattingly 2013; Mcabbe et al. 2013; USFWS 2015). The USFWS (2015) recovery plan for BSD states that further investigation analyzing the link between elevated conductivity and BSD at all life history stages is critical to the conservation of this species (USFWS 2015; Mattingly and Floyd 2013).

The goal of this project is to further elucidate proximate explanations for the progressive extirpation and contemporary genetic disjunction of BSD populations. Using a closely related and ecologically relevant species, *Chrosomus erythrogaster (*Southern Redbelly Dace, SRBD),

we aim to explicitly identify the fitness costs associated with exposure to elevated water conductivity caused by alkaline mine drainage. The use of surrogate organisms for research concerning threatened and endangered species is common practice (Shoffield and Ross 2003), and for our experiments SRBD was used as a surrogate species in place of BSD; furthermore, our study is not the first to use SRBD as a surrogate for BSD in laboratory experiments (Detar and Mattingly 2004). *Chrosomus* is a monophyletic genus containing seven species in North America; SRBD and BSD are within sister clades and represent the only two species of *Chrosomus* with sympatric populations (USFWS 2017; Strange and Mayden 2009). Throughout its range, SRBD inhabits unpolluted 1st to 3rd order streams with low sedimentation and unaltered fish communities (Stasiak 2007) and Mattingly and Black (2013) found that SRBD and BSD share strong preferences for similar habitat in Kentucky. We hypothesize that high conductivity water affects reproduction of SRBD and causes sub-lethal effects which contribute to reduced fitness in adults. We predict a reduction in SRBD egg hatch rate as water conductivity increases as well as a positive correlation between increased water conductivity and increased acute stress in adult SRBD. Additionally, we predict reduced growth in SRBD adults exposed to high conductivity water compared to control water and expect HPI axis dysfunction to be a secondary stress characteristic observed in chronic exposures.

II. Methods

A. Acute Stress: Egg Hatching Experimentation

i. Fish collection and maintenance

Southern Redbelly Dace in Tennessee begin spawning around mid-March and will spawn intermittently until mid-June (Settles and Hoyt 1978). Between May 1st, 2019 and May 17th, 2019, 23 wild SRBD were caught via seine at two localities within the Cumberland River

watershed for captive propagation: Gause Rd. tributary of Miller's Creek (36.442559 N, -87.034027 W) Robertson County, TN and Dry Fork Creek (36.26667 N, -86.85966 W) Davidson County, TN. After collection, we immediately transported fish from field sites and evenly divided them into separated groups in two 40-gallon spawning tanks to prevent overcrowding.

ii. Induction of captive spawning

The manipulation of photoperiod, diet, and water temperature can successfully induce spawning in other closely related minnow species (Rakes et al. 2013) and proved to be equally influential for SRBD. Wild SBRD are associated with the gravel nests of other minnows such as *Campostoma oligolepis* and *Semotilus atromaculatus* (Settles and Hoyt 1978). Our captive SRBD spawned without any physical manipulation of the gravel substrate; however, we found that construction of a mound 30 cm x 30 cm with pebbles at least 5 cm in diameter vastly improved egg recovery by providing a refuge for the demersal eggs from adult SRBD that will readily eat exposed eggs. We controlled photoperiod via 24-hour timer to produce a 13.5/10.5 hr. light/dark cycle. The intensity of light remained static throughout the 13.5-hour window.

We measured water temperature of streams containing spawning SRBD populations seven times between May and June of 2019. Within- stream temperatures ranged from 17-19 °C with a steady, positive increase across months. Based on our observations of fish during four documented spawning episodes, captive spawning occurred when the tank water was at a temperature between 20 and 21 °C. Water temperature was regulated by manipulating the ambient air temperature of the laboratory coupled with placement of a 100 watt heater inside the aquarium. Once temperatures remained stable for more than five days and no spawning behaviors (changes in coloration of pelvic fins, pectoral fins, and ventral half of fish as well as "chasing" behavior among individuals) were observed, a 30% cool water change was performed

to decrease tank temperature by approximately 1.5-2.5 °C. This method proved sufficient to induce spawning on multiple occasions. Fish were fed frozen bloodworms, Mysis shrimp, and Spirulina twice a day while in spawning condition.

iii. Collection and distribution of eggs

We collected eggs immediately after spawning and egg release were observed. Eggs were collected via gentle disturbance of substrate by gravel-vac and filtered from water through a fine mesh dip net (1/32 inch) suspended (<2 inches) in water. We removed eggs from the net by turning net inside out and dipping into 500 ml plastic container holding 200-300 ml of tank water. Individual eggs were then transferred via modified 5 mL transfer pipet (enlarged opening) from the holding container to plastic petri dish holding 50 mL of tank water. We assessed eggs under dissecting microscope and dead or damaged eggs (opaque color or containing incomplete yolk) were removed and viable eggs were randomly distributed to treatments. Individual test chambers consisted of an 800 cm^3 square, polycarbonate container with a 150 mm x 15 mm polycarbonate petri dish placed inside (Figure 1a). Chambers were filled with 400 mL of treatment water and eggs were placed within the petri dish. We provided aeration via vinyl airline tubing placed in the corner of the chamber and set to produce ~1 bubble/second.

iv. Preparation of reconstituted water

One of the primary goals of this study was to create reconstituted water chemically analogous to both impaired and reference streams within BSD range of distribution. For our egg-hatching experiment, we chose to use one control and three (low, medium, and high) treatments that spanned the documented range of stream conductivity and major ion concentration characteristic of reference and mining impaired streams within BSD historic range and relevant, adjacent

ecoregions (Table 1). Water parameters for our low conductivity treatment were based on data provided by Kentucky Division of Water from sites containing BSD populations. These reference sites are characterized by specific conductivity between 60-100 µS/cm, hardness between 20-40 mg/L $CaCO_3$, between 20-40 mg/L SO_4^{2-}, and below 15 mg/L Ca^{2+}/Mg^{2+} (KY Division of Water, personal communication). The target water quality parameters for our medium conductivity and high conductivity treatments were derived from open-source stream monitoring data in the Upper Cumberland River basin and published research on the water quality of surface mining impacted sites in EPA Ecoregions 68, 69, and 70 (Kunz et al. 2013; USEPA 2012; USEPA 2011a; KDOW 2008; Pond et al. 2008). Our medium conductivity treatment water was targeted to have a specific conductivity between 650-750 µS/cm, hardness 250-350 mg/L $CaCO_3$, between 250-350 mg/L of SO_4^{2-}, between 40-60mg/L Ca^{2+}/Mg^{2+}, and below 10 mg Cl^- and K^+. Target parameters for our high conductivity treatment are: specific conductivity between 1500-1650 µS/cm, hardness 550-750 mg/L $CaCO_3$, between 650-850 mg/L SO_4^{2-}, between 100-150mg/L Ca^{2+}/Mg^{2+}, and 15 mg/L Cl^- and K^+. Parameters for control (dilution) water reflected water quality measured at four SRBD collection sites in spring 2019 (Table 2). These sites had conductivity ranging from 250-300 µS/cm, hardness 70-100 mg/L $CaCO_3$, SO_4^{2-} between 50-100 mg/L, Ca^{2+}/Mg^{2+} between 10-20 mg/L, and below 10 mg Cl^- and K^+. We created 20-liter batches of concentrated treatment water and dilution water from a mixture of $MgSO_4$, $NaCO_3$, KCl, and $CaSO_4$ salts and deionized water and allowed each batch to aerate overnight to ensure sufficient dissolution of salts. Water was stored at 4 °C and new batches were mixed every two weeks. Water for controls and each treatment were created, in bulk, from a mixture of dilution, concentrated, and deionized water to achieve predetermined parameters.

Major cations, anions, pH, and conductivity were measured in each treatment batch before each egg exposure trial. Dissolved oxygen, conductivity, and ammonia were checked daily for each exposure replicate; and water was renewed by 30% daily. Eggs were checked each day under dissecting microscope for signs of infection or death, and affected eggs were removed. Hatching occurred within five days, after which post-hatch larvae were transferred to separate, larger tanks. Conductivity, pH, D.O., chloride, and potassium ions were determined with Vernier probes and analyzed using LoggerPro3 software (v. 3.15, Vernier). Ammonia levels were assessed using ammonia test kit drops (API Aquarium Pharmaceuticals). Sulfate, total hardness, and total alkalinity were measured with Hach test kits (alkalinity-Model AL-DT; sulfate- Model SF-1; hardness- Model HA-71A). Calcium and magnesium concentrations were measured via Vernier probes and atomic absorbance spectrophotometry.

B. Acute Stress: Cortisol Experiments

i. Acute Experiment 1: consecutive day sub-lethal testing

We measured acute, sub-lethal effects of conductivity-induced stress to SRBD via analysis of cortisol release rates of wild-caught, experimentally naïve fish in two separate experiments between December 2019 and March 2020. Cortisol diffuses across the gills and into the surrounding environment in levels reflecting circulating plasma concentrations, which can be measured directly as a less invasive alternative to plasma extraction and has been validated for use in fish across taxonomic groups (Crovo et al. 2015; Gabor and Contreras 2012; Archard et al. 2012; Scott and Ellis 2006). Water treatments for both experiments consisted of a control, a medium conductivity reconstituted water, and a high conductivity reconstituted water (control, moderate, high). In our Acute, sub-lethal experiments, we did not expose fish to the low conductivity treatment used in egg-hatching experimentation. Control, medium, and high

conductivity treatment waters for cortisol tests were created and stored similarly to reconstituted water used in egg-hatching tests (Table 1). We measured alkalinity, hardness, and sulfate concentrations for each treatment batch prior to fish exposure and measured dissolved O_2, pH, and conductivity of individual exposure beakers immediately prior to fish placement. In the first experiment, we exposed each SRBD (n = 9) to the control, medium, and high conductivity treatment water over the course of three consecutive days. Each fish was exposed to a different treatment each day so that all fish were exposed to each treatment once, and the order of exposure was randomized. We held fish in groups of three within 20-gallon aquaria for acclimation 48 hours prior to testing. The grouping of fish was necessary for identification in order to prevent groups from being tested in a single treatment water more than once; furthermore, SRBD are highly social in the wild, and isolation of individual fish during holding would exacerbate stress. Fish were fed flakes (Ken's Premium Tropical Flake, Taunton, MA) once daily and provided a 12L:12D photoperiod during acclimation.

ii. Acute Experiment 2: single day sub-lethal testing fish use

In order to remove confounding effects of multiple-day exposure on cortisol release rate, we performed a second acute exposure experiment in which a second set of experimentally naïve SRBD (n = 12) were each only exposed to one treatment water type on a single day so that 4 fish were tested in control, medium, and high conductivity treatments. Fish were placed into a single 20-gallon aquarium for 48-hour acclimation prior to testing, fed flakes (Ken's Premium Tropical Flake, Taunton, MA) once daily, and provided a 12L:12D photoperiod during acclimation. Reconstituted water use, experimental procedure, and methods for cortisol extraction were the same for both Experiments 1 and 2 and are described below.

iii. Experimental protocol and cortisol collection

We performed all experimental trials for our Acute Cortisol Experiments between 10:00 AM and 11:00 AM to account for daily hormonal fluctuations. We placed individual test fish in 500 mL beakers containing 300 mL of test solution. Beakers were positioned side by side within glass 13 cm x 13 cm glass compartments half filled with water so that fish were visible to each other in order to reduce stress caused by isolation (Figure 1b). In Acute Experiment 1, dissolved oxygen concentrations were measured for all units immediately before introducing test subjects using a calibrated, Clark-type polarographic electrode (Vernier; DO-BTA, range: 0-15 mg/L; accuracy: ± 0.2 mg/L). Fish were placed in their randomly assigned treatment water and remained in the treatment for 30 minutes. Fish begin releasing metabolized steroids from gill lamella into the water within minutes of exposure to stressor, and 30 minutes is sufficient time for fish to respond to acute stress without inducing hypoxic-related stress (Flik et al. 2006). After 30 minutes, fish were weighed individually and placed into holding tanks with addition of Stress-ZYME (API Aquarium Pharmaceuticals). Immediately after testing, we measured dissolved oxygen and filtered the water through C-18 cartridges (Sep-pak, Waters Technology Corporation, Milford, MI) primed with 4 mL of methanol and pumped at 25 mL/minute using a vacuum manifold. Cortisol was eluted from filters with 5 mL ethyl acetate into glass test tubes and evaporated under pressurized air. After evaporation, residue was either immediately re-suspended or stored at -80C until re-suspension (<72 hours). We re-suspended dried residue in 350 µl of ELISA buffer and the suspension was assayed at 1:200 dilution. The plate was incubated, developed, and read at a wavelength of 415 nm according to procedures outlined in Cortisol ELISA instructions (Cayman Chemical, Ann Arbor, MI). ELISA kits were validated for SRBD before analysis by serially diluting a pooled sample consisting of 10 non-experimental

fish and achieving parallelism with standard curve provided with ELISA kit (slope comparison; $p = .99$). A second set of serially diluted samples pooled from 10 non-experimental fish were spiked with known concentrations of cortisol to assess recovery. Blanks were run each treatment water and subtracted from measurements to account for assay cross-reactivity with background water. We measured differences in dissolved oxygen concentrations before and after testing by suspending the probe in 6 cm of test beaker water and slowly swirling to homogenize the treatment solution. Oxygen measurements were taken at 30 seconds to ensure stabilization of reading. Blank dissolved oxygen concentrations were measured in each treatment without fish to account for background changes in dissolved O2 over 30-minute trial time.

C. Chronic Stress: Growth and HPI Testing

i. Growth experiment

We assessed the effects of chronic stress on 12 experimentally naïve adult SRBD by tracking weekly weight differences in groups of fish exposed to either a control or highly impaired treatment water over the course of five weeks. Fish were held in groups of three, and we exposed two groups to control water and two groups to impaired water for the duration of the experiment. The variation in starting weight among individuals of a group was <10% and the variation in total weights between groups was <10%. Mean mass of individual fish (n=12) was 1.61g and mean mass of each group (4 total) was 4.83g. All fish were fed 0.3 g flake food (Ken's Premium Tropical Flake, Taunton, MA) Monday, Wednesday, Friday; fed 0.15 g Tuesday, Thursday, Saturday, and fasted Sunday. We constructed two 20-gallon control tanks using dechlorinated tap water to minimize any chemical variation occurring between control and previous holding tank. Tap water at Austin Peay State University is moderately hard, between 300-320 μS/cm, and has a pH of roughly 7.5-7.8. We created water used for the two impaired-

treatment tanks using salt concentrations outlined for the concentrated impaired water used in the egg-hatching experiments. Every seven days, we weighed each group separately in a 300 mL beaker containing 150 mL of tank water. We recorded weights of each group to the thousandth of a gram after all three fish were in the beaker. After weights were taken, fish were returned to their experimental tanks with the addition of Stress-Zyme.

ii. HPI axis assessment

Using the 12 SRBD from the growth experiments, we measured individual cortisol release rates after application of an acute stressor to test for evidence of hypothalamic-pituitary-interrenal (HPI) axis dysfunction. We held the fish in the same water they had been exposed to during the growth experiment for one additional week prior to cortisol tests in order to reduce stress related to previous handling/weighing; therefore, at the point of testing, each fish had been exposed to experimental conditions for a total of six weeks. At the start of each trial, we placed each fish in a 1000ml beaker containing 400ml of dechlorinated tap water and immediately used small dip-nets to chase each fish for the first 30 seconds within the beaker, while avoiding direct contact. After being chased for 30 seconds, fish were left alone for one hour and removed from testing beakers. We filtered test water immediately after one-hour exposures using primed C-18 cartridges. All methods for hormone extraction and analysis of cortisol were the same as methods used in previous cortisol tests.

III. Statistical Analyses

All statistical analyses were performed in R (version 4.0; R Development Core Team 2020) and we visually inspected datasets for compliance with individual test assumptions using "autoplot" function (package GGfortify, Horikoshi et al. 2020). If assumptions of linear

regression analyses were not met, we used Kruskal-Wallis tests in place of ANOVA or generalized linear models (GLM) and "boxcox" function (package; MASS) to guide decisions on data transformation. For egg-hatching experimentation, we assessed the probability of egg mortality based on water conductivity using a weighted, binomial GLM. Egg hatching data did not meet normality assumptions, therefore we used Kruskal-Wallis with post-hoc Dunn's test to analyze differences in egg-hatch proportions among treatments.

The data from the first two acute cortisol experiments were mass adjusted and expressed as release rate (ng g^{-1} $30min^{-1}$) per individual. Our results were unbalanced after the loss of a sample and did not meet the homoscedasticity assumption of a repeated-measures ANOVA, therefore we fit the data from Acute Experiment 1 (consecutive day tests) into log-linked, repeated-measures Generalized Linear Mixed Model (GLMM; package lme4, Bates and Maechler 2010) and made post-hoc pairwise comparisons using estimated marginal means (EMM; package "emmeans" v. 1.4.7, Length et al. 2020). We included treatment and day as within-subject factors and included subject ID as random effects in our model. We structured models a-priori to assess how interactions between conductivity and the specific day in which fish were tested influenced cortisol release rate and performed subsequent model comparisons using log-likelihood ratio tests. We analyzed oxygen consumption among treatments via repeated measures ANOVA with post-hoc Tukey-test. For Acute Experiment 2 (single day testing), we used a one-way ANOVA to assess cortisol release rate among treatments and post-hoc Tukey test to compare cortisol release rates between treatments. Oxygen consumption data from Acute Experiment 1 was mass-adjusted for fish weight and expressed as mg/L O_2 g^{-1} $30min^{-1}$.

We assessed the effect of prolonged exposure to elevated water conductivity on group weights in our chronic growth experiment using a mixed-model ANOVA to account for

autocorrelation. We used treatment as a between-subject factor, time as within-subject factor, and we added group ID as a random effect. We made post-hoc comparisons using EMM to identify specific time points (week 0-5) where significant weight differences were present between high conductivity and control treatments. We analyzed subsequent HPI functionality after chronic exposures using log-transformed cortisol release rates (ng g^{-1} $60min^{-1}$) to meet normality assumptions and compared release rates between high conductivity and control treatments using a two-sample Student's T-test.

IV Results

A. Egg Hatching Tests

i. Reconstituted water parameters

A major challenge in toxicology experiments using reconstituted water is ensuring the ionic composition of treatments accurately falls within target ranges. We took all necessary precautions to mitigate significant deviations by calibrating equipment multiple times, using precise measurements of salt masses and water volumes, and allowing sufficient time for salt dissolution; however, the inherent characteristics of interacting dissolved major ions make 100% target accuracy difficult to achieve. The chemical parameters of the control, low, and two elevated conductivity waters used for egg-hatching tests were created to reflect a range of mining-related degradation found throughout BSD historic range. The mean water quality measurements across all egg exposure trials fell within our target levels (Table 3). The specific conductivity, pH, chloride, potassium, calcium, total alkalinity, DO, and ammonia never measured outside of target parameters for any treatment. It is also important to note that no ionic deviations occurred outside the documented range of field-measured, mining impaired streams.

ii. Egg-hatching results

In October of 2019, we collected over 700 eggs within 6 hours of fertilization from a holding tank containing seven wild caught SRBD. The average proportion of eggs hatched among all treatments was 81.75%, and mean hatch proportions for each treatment were: control 93%, low 94%, mid 71%, and high 69% (Table 4). Increasing water conductivity significantly decreased the probability of hatching success (GLM, $SE = 0.00018$, $z = 6.29_{22.6,\ 10}$ $p = <0.001$), and with an increase in water conductivity from 100 µS/cm to 1650 µS/cm, the estimated probability of hatching decreased by 27% (95% CI: 5%-54%). There were significant differences in proportion hatch between the low/control treatments and high conductivity treatment (Kruskal-Wallis post-hoc Dunn's; z = 2.106, $p = 0.035$, $n = 12$) as well as low/control vs. medium conductivity treatment (Kruskal-Wallis post-hoc Dunn's; z = 1.99, $p = 0.046$, $n = 12$, Figure 2). There was a total of 101 egg mortalities across all treatments, with 70% of mortality occurring within the first 48 hours of exposure to treatment (Figure 3 a-d); 95% of surviving eggs hatched within 5 days.

B. Acute Sub-lethal Exposure Results

ii.. Acute Experiment 1: consecutive day tests

In Acute Experiment 1, we randomly exposed 12 adult SRBD for 30 minutes to control (278-287 µS/cm), medium conductivity (830-850 µS/cm), and high conductivity (1675-1690 µS/cm) treatments over three consecutive days and measured the cortisol release rate of individual fish. Across the three day trial, mean cortisol release rate in control water was 4.84 ng g^{-1} $30min^{-1}$ ($SD = 2.198$), in medium conductivity water was 5.23 ng g^{-1} $30min^{-1}$ ($SD = 2.95$), and in high conductivity water was 3.91 ng g^{-1} $30min^{-1}$ ($SD = 1.47$; Figure 4). The pooled cortisol release rates of SRBD in Acute Experiment 1 were not significantly different among

treatment waters (GLMM, EMM; control-moderate, *SE* = 0.907, *p* = 0.60; control-high, *SE* = 0.27, *p* = 0.44; moderate-high, *SE* = 0.28, *p* = 0.21) and water conductivity alone was not a significant explanatory variable in the amount of cortisol release across the three day experiment (GLMM; *t* = 1.09, *p* = 0.27*)*. However, cortisol release rate was significantly influenced by the interaction between the conductivity of treatment water and the day in which groups were tested. On day one, the highest cortisol release rate was among fish exposed to high conductivity water (5.91 ng g^{-1} $30min^{-1}$, *SD* = 0.39). Increasing conductivity on days two and three were associated with decreases in cortisol release rate (GLMM; Day 2, *t* =-2.21, *p* = 0.027; Day 3, *t* = -1.28, *p* = 0.17, Figure 5). Our model predicted a 5.91 ng g^{-1} $30min^{-1}$ (SE± 1.41) cortisol release rate for fish exposed to high conductivity treatment water on day one, 2.11 ng g^{-1} 30min (SE ± 1.74) for fish exposed high conductivity day two, and on day three cortisol release rate in high conductivity water was predicted lowest at 1.99 ng g^{-1} 30min (SE ± 1.8).

Dissolved oxygen concentration was measured before and after every test in Acute Experiment 1. The highest average oxygen consumption was measured in fish exposed to high conductivity water (0.55 mg/L/30 min, *SD*= 0.05) followed by fish exposed to medium conductivity water (0.45 mg/L/30 min, *SD* = 0.01) with fish in the control water consuming the least amount of oxygen (0.37 mg/L, *SD* = 0.06). Treatment type significantly explained differences in oxygen consumption (ANOVA; $F_{2,17}$ = 11.62, p < 0.001) and oxygen consumption was significantly different between control and high treatments as well as moderate and high treatments (Tukey test; control-high, *p*<0.001; mid-high, *p* = 0.028, *n* = 27, Figure 6), with no differences detected between days (Tukey test, days 1-3 *p=0.*99). The mean intra-assay coefficient of variation from Acute Experiment 1 was 13% and inter-assay variation was not calculated since all samples were assayed on one plate.

ii. Acute Experiment 2: single day testing

In Acute Experiment 2, in which 12 adult, naive SRBD were exposed a single time to either control, medium conductivity, or high conductivity water, we detected significant differences in cortisol release rate among treatments (ANOVA; $F_{2,9} = 7.34$, $p = 0.013$, $n = 12$) and significant differences in cortisol release rate between the control and highly conductivity treatment (Tukey test; $p = 0.01$, Figure 7). Fish in high conductivity treatment released, on average, the most cortisol (3.99 ng g^{-1} $30min^{-1}$, $SD = 0.98$) followed by fish exposed to medium conductivity water (2.72 ng g^{-1} $30min^{-1}$, $SD = 0.26$) and those exposed to control water had the lowest average cortisol release rate among the treatments (2.07 ng g^{-1} $30min^{-1}$, $SD = 0.21$). Oxygen consumption was not measured for fish in Acute Experiment 2. Water quality measurements for both acute cortisol experiments fell within target values (Table 5). The cortisol samples were analyzed on one plate and the mean intra-assay coefficient of variation for Acute Experiment 2 was 10.8%

C. Chronic 6 Week Growth Study and HPI Axis Results

i. Fish growth over 5 weeks

In our chronic growth experiment, we exposed six adult SRBD to control water (two groups of three fish) and six adult SRBD to high conductivity water (two groups of three fish) for five weeks and tracked the weekly weights of each group of fish. The average starting weights for control groups was 4.9 g (Control group 1, 4.97g; Control group 2, 4.87g) and average starting weights for high conductivity groups was 4.71g (High group 1, 4.96 g; High group 2, 4.60 g). At the conclusion of week five the control groups had gained an average of 1.2 g (final weights: Control group 1, 5.91g; Control group 2, 6.36 g; EMM; $t = -4.814$, $p =$

0.006), a 25% increase in weight since initiation whereas high conductivity groups gained an average of 0.26 g, a 5% increase from start (final weights: High group 1, 5.08g; High group 2, 4.92g; EMM; t = -1.034, p = 0.89, Figure 8). Treatment type was a significant predictor of weight differences between groups over the course of the experiment (ANOVA; $F_{1,18}$ = 7.11 , p = 0.016, 95% CI: -0.27, -0.04). Accounting for individual group variation, we found significant differences in weights between treatment and control groups each week from weeks two-five (EMM; week 2, p=0.023; week 3, p=0.01; week 4, p=0.01; week 5, p=0.003, Figure 8). The mean conductivity of control water over the six weeks was 390 μS/cm and mean conductivity of high conductivity water was 1680 μS/cm.

ii. Subsequent cortisol release rate of SRBD after growth study

One week after the conclusion of the previous growth experiment we tested the cortisol release rates of all 12 SRBD. We provided an acute stressor by chasing fish for 30 seconds in a beaker and measured cortisol release rates after one hour. The average cortisol release rate for fish tested in control water was 6.96 ng g^{-1} 60 min^{-1} (n = 6, SD = 4.6) and the average cortisol release rate of fish after chronic high conductivity exposure was 4.03 ng g^{-1} 60 min^{-1} (n = 6, SD = 2.28). Despite a 53% difference between the two group means, we did not detect a statistically significant difference between the two groups of fish (T-test; 95% CI: -0.11, 0.56; p = 0.16) driven by the high variation among group release rates (Figure 9).

VI. Discussion

Acute egg toxicity

In our egg-hatching experiment we found that exposure to reconstituted mining-impaired water above 650 μS/cm significantly reduced hatching success of newly-fertilized (<24hr) embryonic SRBD compared to low conductivity and control treatments. Our study is the first to

document acute mortality of fish eggs resulting from exposure to alkaline mine polluted water; and our observed toxicity thresholds for SRBD embryonic mortality are consistent with survival thresholds observed for Appalachian macroinvertebrates to alkaline, mine-polluted water (Kunz et al. 2013; Passmore et al. 2008) and major ion toxicity to fathead minnows (Wang et al. 2008). Since aquatic macroinvertebrates and fish share numerous, identical ionic exchange pathways to maintain homeostasis, acute toxicity of aquatic macroinvertebrates to mining impaired water are informative where fish-derived thresholds are lacking (Evans 2009). For example, experiments using reconstituted water similar to our medium and high conductivity treatments (high hardness dominated by Ca, Mg, and SO4 ions) documented 82% mortality in mussels (*Lampsilis silquoidea)* exposed to water above 500 µS/cm, and 70-100% mortality for mayfly (*Hyella Azteca)* in water above 900 µS/cm (Kunz et al., 2013).

In freshwater fish embryos, osmoregulation is maintained through mitochondria-rich ionocytes within the skin prior to gill functionality (Guh, Lin, & Hwang 2015). The primary co-transport activities of embryonic skin ionocytes function similarly throughout ontogeny and regulate Na, Ca, and Cl uptake as well as H, NH_4, K, and HCO_3 secretion (Dyomowska et al 2012). Exposure to increased concentrations of major cations and anions, particularly for a stenohaline fish adapted to low salinity (such as BSD and SRBD) presents physiological challenges that may ultimately end in death (USEPA 2011a). Characterizing the toxicity of a solution to fish embryos is challenging, in part because differing concentrations of co-occurring ions also vary in their toxicity (Mount et al. 2019), and because tolerance to salinity in embryonic stages varies widely across species (Myosho et al. 2018; Weber-Scanny & Duffy 2007). Consequently, results from acute toxicity tests using the same species may report different effect concentrations depending on salt mixtures used and the characteristics of dilution water

(Mount et al. 2019; Weber-scanny & Duffy 2007). Our reconstituted, medium and high conductivity treatments closely matched the target parameters derived from published field measurements in mining-affected streams, and the dominant major ions in our high conductivity treatments were HCO_3, SO_4, Ca, and Mg. Elevated SO_4 and HCO_3 both appear to be important contributors to the acute toxicity of fathead minnow (*P. promelas)* larvae and adults (Wang et al. 2016; Mount et al. 1997). Our elevated conductivity treatments ranged from 280-985 mg/L SO_4, which is within range of LC50 SO_4 concentrations observed for < 24hr embryos of *P. promelas* in 7-day exposures (Wang et al. 2016); however, the toxicity of SO_4 seems to be ameliorated with both increased hardness and increased K^+ concentrations (Wang et al. 2016; Lasier and Hardin 2010). The proportional increase in hardness and K^+ concentration between our moderately and high conductivity water may explain why the hatch rates of SRBD did not differ significantly between the two treatments despite a ~900 μS/cm increase in conductivity (Table 3).

The timing between specific events such as egg fertilization, egg collection, treatment exposure, and experimental conclusion appear to influence results of toxicity experiments using embryonic fish. Longer exposure periods using hatched larvae for up to 35 days have been demonstrated to yield increased toxicity in fathead minnows compared to 7-14 day trials (Wang et al. 2016). Additionally, eggs exposed to treatments earlier appear to be more sensitive to toxicants, which may relate to a threshold within an embryonic critical period, after which tolerance to salinization increases (Wang et al 2016; Skar et al. 2006). During this critical period, the development of larval fin rays, vertebrae, myotomes, and gill rakers is influenced by environmental factors (Helfman et al. 2009). In future studies the post-hatch measurement of

meristic traits as well as documentation of visible abnormalities in embryonic development should be considered as supplemental quantification of sub-lethal stress.

It is likely that our egg-hatching results are a conservative reflection of the direct fitness costs to wild BSD associated with surface mining activity. We collected 103 viable eggs per breeding SRBD adult in the October 2019 spawning event, which is over three times greater than the reported number produced for any BSD captive propagation event (Rakes et al. 2013; 1992). We gathered eggs within 12-24 hours of fertilization and concluded the test after seven days; exposures started earlier than 12 hours post-fertilization may lead to even higher egg mortality than was observed in our experiment (Skar et al. 2006). Increased water conductivity may also disrupt osmoregulatory mechanisms of eggs during external fertilization, since ionic transport processes drive membrane permeability for externally fertilized fish eggs (Tarin et al. 2000). Exposure to elevated conductivity at fertilization has been demonstrated to decrease hatch rate in Coho Salmon compared to eggs exposed post-fertilization (Stekoll et al. 2003).

Sub-lethal stress

We also present evidence of sub-lethal stress in adult fish resulting from acute exposure to alkaline, high conductivity water. Although no known studies have investigated fishes' response to our system of interest, our results are consistent with literature reporting acute stress in freshwater ostaryophysans via waterborne hormone collection (Crovo 2015; Wysocki et al 2005) as well as acute cortisol increase in ostaryophysans to increased salinization (Babershke et al. 2019; Irob et al. 2019).

On the first day of Acute Experiment 1, we observed a trend indicating increased cortisol release as water conductivity increased (Figure 5). This observation was verified in Acute

Experiment 2 by our detection of significantly increased cortisol release from SRBD in the high conductivity treatment compared to low and moderate treatments (Figure 7). Secondary responses to stress may also manifest as increased respiration rate and elevated gill permeability to support the increased metabolic demand (Barton et al. 2002), which are reflected in the significantly increased oxygen consumption we measured from fish exposed to the high conductivity treatment in Acute Experiment 1 (Figure 6). The differences in oxygen consumption we detected in Acute Experiment 1 also follow the pattern of cortisol release rates observed in day one of Acute Experiment 1 and Acute Experiment 2, all of which demonstrate an acute stress response to elevated water conductivity. In fish, cortisol functions as a catalyst for the recruitment of mitochondrial-rich epithelial cells to gill lamella in order to facilitate short-term management of osmoregulatory disruption (Evans et al. 2005). This process may partially explain the overall increased oxygen consumption we detected among fish exposed to high conductivity treatments across all three days despite significantly reduced cortisol release in the same high conductivity treatment on days two and three of Acute Experiment 1. Nonetheless, the results from our Acute Experiments 1 & 2 demonstrate that elevated water conductivity analogous to alkaline, mine-impaired streams causes measurable, sub-lethal stress to adult SRBD, and likely effect BSD similarly.

Additionally, we detected evidence of chronic stress as reflected by significantly reduced growth rates in SRBD compared to control groups over a five-week exposure. Similar studies have documented reduced biomass in fish after chronic exposure to abnormally saline water (Wong et al. 2008; Babershke et al. 2019). Persistent interrenal activation resulting in HPI dysfunction has been widely documented in fish exposed to chronic stress caused by environmental contamination and presents itself as reduced corticosteroid response to additional

stressors (Marentette et al. 2013; Laflamme et al. 2000; Norris et al. 1999; Hontela 1997). We tested for HPI dysfunction by applying an acute stressor and measuring subsequent cortisol release rates of all fish from chronic growth experiment. On average, the high conductivity group released less cortisol; however, due to large group variation in release rates, our results were not significant. Our chronic experimentation was limited by sample size, and adding fish or using additional biometrics affected by HPI dysfunction such as histopathology of specific organs, circulating leukocyte densities, or measurement of ventilation rate may have revealed significant differences between groups and should be considered in future studies (Helfmann et al. 2009). Nonetheless, we have evidence that prolonged exposure to high conductivity, alkaline mine water causes chronic stress in adult SRBD.

Reduced growth is only one response to chronic stress, and many other important biological functions are detrimentally affected (Helfmann et al. 2009; Barton 2002). Persistent stress has been shown to reduce immune function in fish by the induction of B-cell apoptosis (Weyts et al. 2003), inhibition of inflammatory responses (Saeji et al., 2003), and modification of circulating leukocyte densities (Moijaszek et al. 2002), all of which ultimately lead to decreased disease resistance and increased susceptibility to environmental pathogens (Leupes et al. 2006). Additionally, chronic stress reduces reproductive fitness in fish; specifically, by inhibiting production of sex hormones (Helfmann et al. 2009), reducing gamete and egg quality, and subsequently decreasing growth of larval fish (Weil et al. 2001; McCormick 1998). Lastly, chronic stress affects skin pigmentation in fish, and periods of prolonged stress in trout resulted in the diversion of carotenoids from skin pigmentation to supplement immune function (Backstrom et al. 2013). We did not explicitly quantify SRBD coloration within our study, but we observed visibly less coloration in fish exposed to high conductivity water compared to the

control water. At least 33% of stream fish species in Tennessee alone exhibit red and yellow nuptial coloration produced by carotenoid-dependent chromatophores (Settles and Hoyt 1978, Etnier and Starnes 1993). The costs of modified carotenoid utilization from pigmentation to immune system supplementation may have significant fitness consequences for spawning stream fish in Central Appalachia that should be explored in future studies.

VII. Conclusion and future directions

We have presented the first evidence of the direct fitness costs associated with alkaline, mining-impaired water on a representative fish species at multiple life history stages. First, we detected 20%-30% mortality of SRBD embryos above 600 μS/cm (Table 4). Second, we measured significant increases in the cortisol release rate and oxygen consumption of adult SRBD during exposure to 1600 μS/cm water (Figures 6 & 7). Lastly, we observed the effects of persistent stress to adult SRBD as reflected by significantly reduced growth in high conductivity treatment water compared to control groups (Figure 8). The mortality and sub-lethal stress conductivity thresholds we observed in SRBD are consistent with field studies demonstrating fish community richness decreases between 600-1000 μS/cm in West Virginia (Hitt and Chambers 2014) and corroborate the observed BSD conductivity thresholds between 240-350 μS/cm in Tennessee and Kentucky (Yates 2017; Hitt et al. 2016; Black et al. 2013). Our study also validates the maximum 300 μS/cm benchmark set by the EPA to protect 95% of aquatic life in ecoregions 68, 69, and 70 (USEPA 2011). Our results suggest that the extirpation of intolerant fish species from streams affected by mining could result from a combination of culminating reproductive failures, stream avoidance related to acute osmotic stress, and decreased survivorship resulting from chronic exposure to elevated conductivity and associated major ions. Species' responses to elevated conductivity are context-dependent (Clements and Kotilak 2015),

and since SRBD are more tolerant of water quality degradation and even displace BSD from disturbed streams (USFWS 2015;1988), we expect BSD at all life history stages to be more sensitive to elevated conductivity.

The BSD is only one of many fish species that are similarly imperiled by the effects of surface mining. The Kentucky Arrow Darter (*Etheostoma spilotum)* is equally sensitive to stream degradation and has recently experienced dramatic range reductions related to elevated conductivity caused by surface mining (Yates 2017; Hitt et al. 2016). Within *Chrosomus*, Laurel Dace (*C. saylori),* Clinch Dace (*C. sp. cf. saylori),* and Tennessee Dace (*C. tennesseensis)* all have extremely limited ranges within heavily mined regions in Appalachia (USFWS 2014; White and Orth 2014). Additionally, our results are directly relevant to SRBD populations at the edge of its range that are critically imperiled by habitat alteration and in need of conservation attention (Stasiak 2007). We hope that our results extend to inform policymakers in guiding recovery management of all imperiled species within Central Appalachia.

Aquatic communities are complex, synergistic systems that function via interactions from numerous biotic and abiotic factors. In Central Appalachia, elevated conductivity is one of many interacting components reflecting stream impairment caused by surface mining (USEPA 2011b; Palmer et al. 2010); however, our results suggest that exposure to elevated conductivity alone has fitness costs for fish at multiple life history stages. Future studies should investigate the contribution of specific ions to toxicity; the specific osmoregulatory pathways that are being affected in fish at different life history stages; interspecific and intraspecific variation in salinity tolerance across the Appalachian region; and how variables such as temperature, increased heavy metals, and reduced prey abundance co-vary with conductivity to reduce fitness in fish.

VIII Figures and Tables

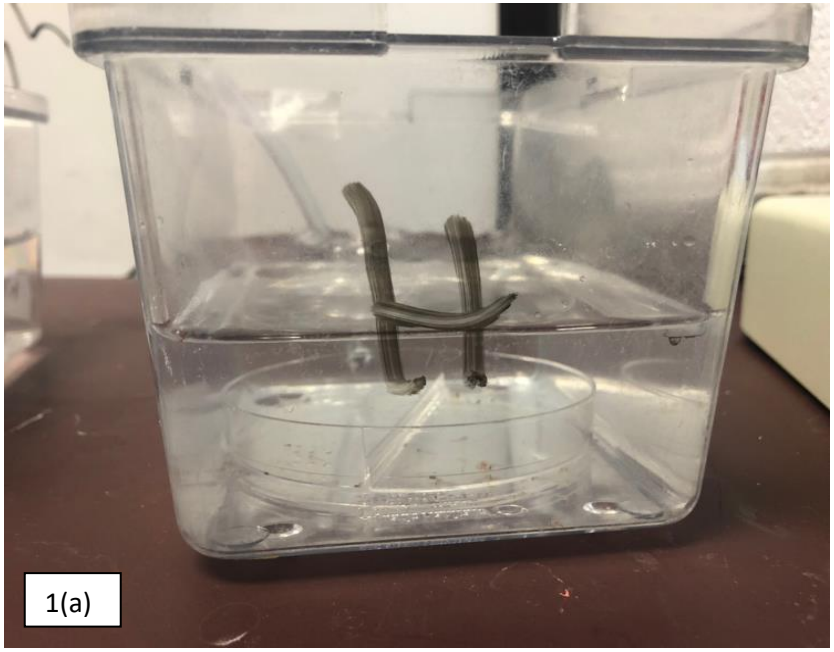


Figure 1(a): Individual, polycarbonate egg-hatch test chamber with <24hr post-hatch larvae. **Figure 1(b)**: Individual test beakers, with adult SRBD, used for static cortisol collection in Acute Experiments 1 & 2. Beakers containing fish were positioned side by side within glass compartments half filled with water so that fish were visible to each other in order to eliminate stress caused by isolation.

Table 1. Target water parameters for control and three treatments used in egg-hatching experiments. Control treatments were derived from field samples taken from SRBD collection sites. Low treatment parameters were provided via field samples collected in BSD-inhabited streams by Kentucky Division of Water[1]; medium and high treatment water were estimated from open source water quality data taken from mining impaired streams in Central Appalachia[2].

	Conductivity (uS/cm)	pH	Hardness (mg/L as CaCO3)	Alkalinity (mg/L as CaCO3)	Sulfate (mg/L)	Calcium (mg/L)	Magnesium (mg/L)	Potassium (mg/L)	Chloride (mg/L)	Ammonia (mg/L)
Control	250-300	7.5-8.5	80-100	80-100	50-100	10.0-20.0	10.0-20.0	<5	1.0-3.0	<.5
Low	60-100	7.5-8.5	20-40	20-40	<50	<10	<10	<5	1.0-3.1	<.5
Medium	650-750	7.5-8.5	250-350	80-100	250-350	40-50	50-60	2.0-6.0	3.0-6.0	<.5
High	1500-1650	7.5-8.5	550-700	120-150	650-850	100-150	100-150	8.0-15.0	8.0-15.0	<.5

[1:] Jacob Culp. 2019. Kentucky Division of Water. Personal communication
[2:]Kunz et al. 2013; USEPA 2011; Pond et al. 2008

Table 2. Water parameters for samples taken at SRBD collection sites (n=4) in Dry Fork Creek, Davidson Co. TN and Miller's Creek trib, Robertson Co. TN. These parameters were used as targets for control water during egg hatching and acute cortisol experiments.

Site ID	Conductivity (uS/cm)	pH	Total Alkalinity (mg/L CaCO3)	Hardness (mg/L as CaCO3)	Calcium (mg/L)	Chloride (mg/L)	Potassium (mg/L)	Sulfate (mg/L)	Magnesium (mg/L)
Miller's Creek	321	7.31	100	99.8	14.2	1.1	0.2	65	10.3
Dry Fork Creek	240	7.70	80	90.6	18.5	0.5	0.5	65	12.3
Miller's Creek 2	288	7.47	100	111.5	11.3	1.0	0.2	50	10.2
Dry Fork Creek 2	251	7.65	100	95.5	20.1	0.5	0.2	50	11.5

Table 3. Water quality characteristics of reconstituted water for egg-hatching experiments. Specific conductivity is presented as mean and standard deviation from measurements before and after trial. Other values are derived from measurements of treatment batches created directly before egg exposure. Concentrations that deviate >20% from target values are noted (*). NA values on first row are due to AA spectrophotometer malfunction.

Treatment	Conductivity (uS/cm)	Temperature (F)	DO (mg/L)	pH	Total Alkalinity (mg/L CaCO3)	Hardness (mg/L as CaCO3)	Calcium (mg/L)	Chloride (mg/L)	Potassium (mg/L)	Sulfate (mg/L)	Magnesium (mg/L)	Mg+Ca:SO4
Control	282.3 (2.51)	68.7	7.8	7.84	90	86	NM	2.2	2.4	70	NM	NM
Low	108 (8.6)	69.7	7.9	7.80	40	34	5.1	1.7	1.5	30	6.3	1.42
Mid	784 (14.1)	68.7	7.9	8.00	100	274	41.7	6.1	5.5	450*	58.9	0.73
High	1636 (35.15)	68.7	7.9	8.21	140	860*	104.6	13.6	14.8	985	170.2*	0.94

Table 4. Number of eggs per treatment, water conductivity (means ± SD taken over seven days), and proportion hatches for egg-hatching experimentation. The egg-hatching experiment consisted of three replicates per treatment, and results are presented as means with standard deviations in parentheses for each treatment (n = 600 eggs from 7 wild-caught SRBD).

Treatment	Conductivity (uS/cm)	Number of eggs at start	Number of mortalities	Proportion hatched (%)
control	282 (3)	50	3 (1)	94 (2)
low	108 (14)	50	3 (1.5)	94 (3)
medium	784 (14)	50	15 (3.4)	71 (6)
high	1636 (35)	50	15 (3)	70 (6)

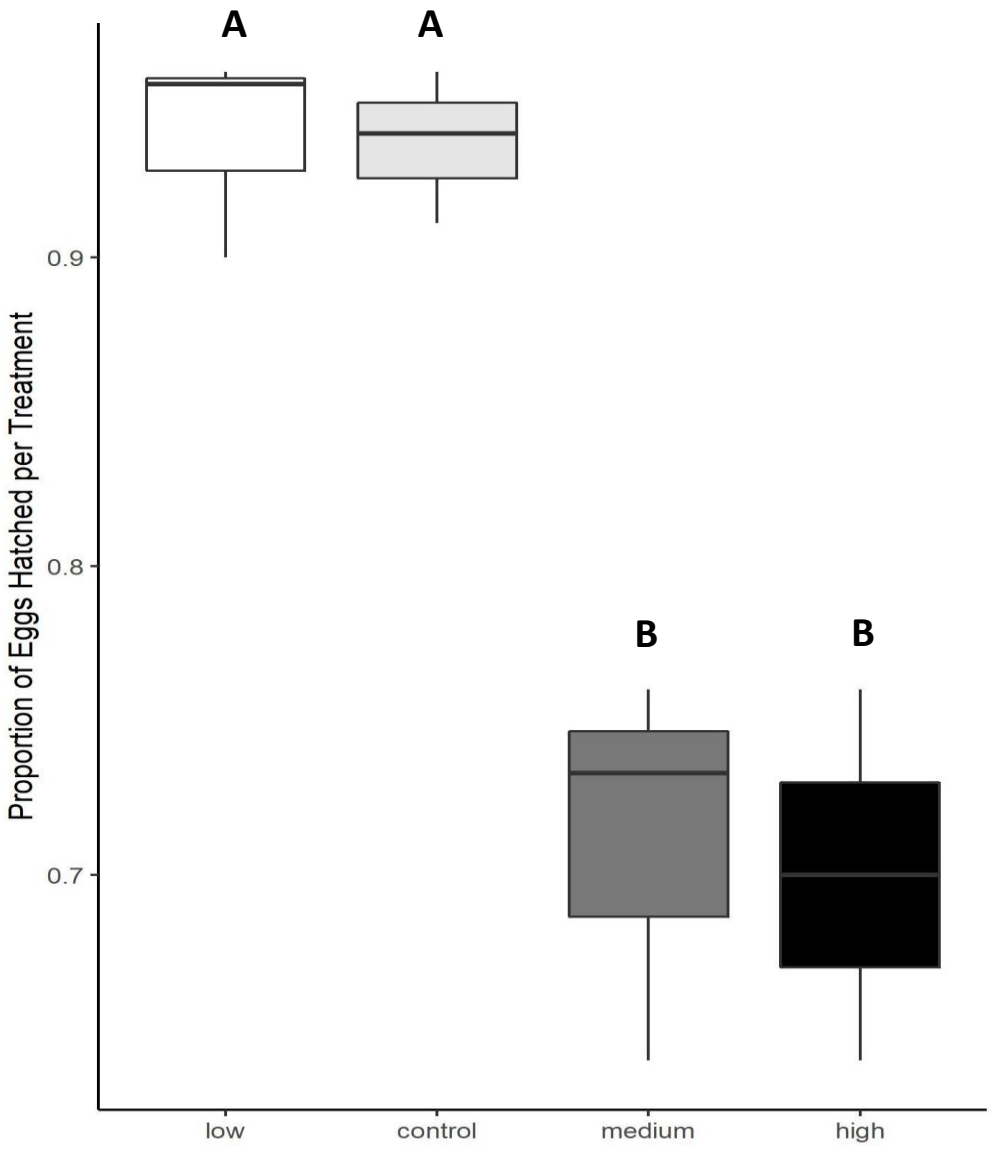


Figure 2. Proportion of eggs hatched among three conductivity treatments in egg-hatching experiment (control 90-125 µS/cm; mid 750-790 µS/cm; high 1620-1665 µS/cm). Significant differences in hatch rates were recovered between low/control vs. high treatments (Kruskal-Wallis post-hoc Dunn's; p=0.035) and low/control vs. medium treatments (Kruskal-Wallis post-hoc Dunns; p = 0.046, n = 600 eggs), indicated by differing letters above plots.

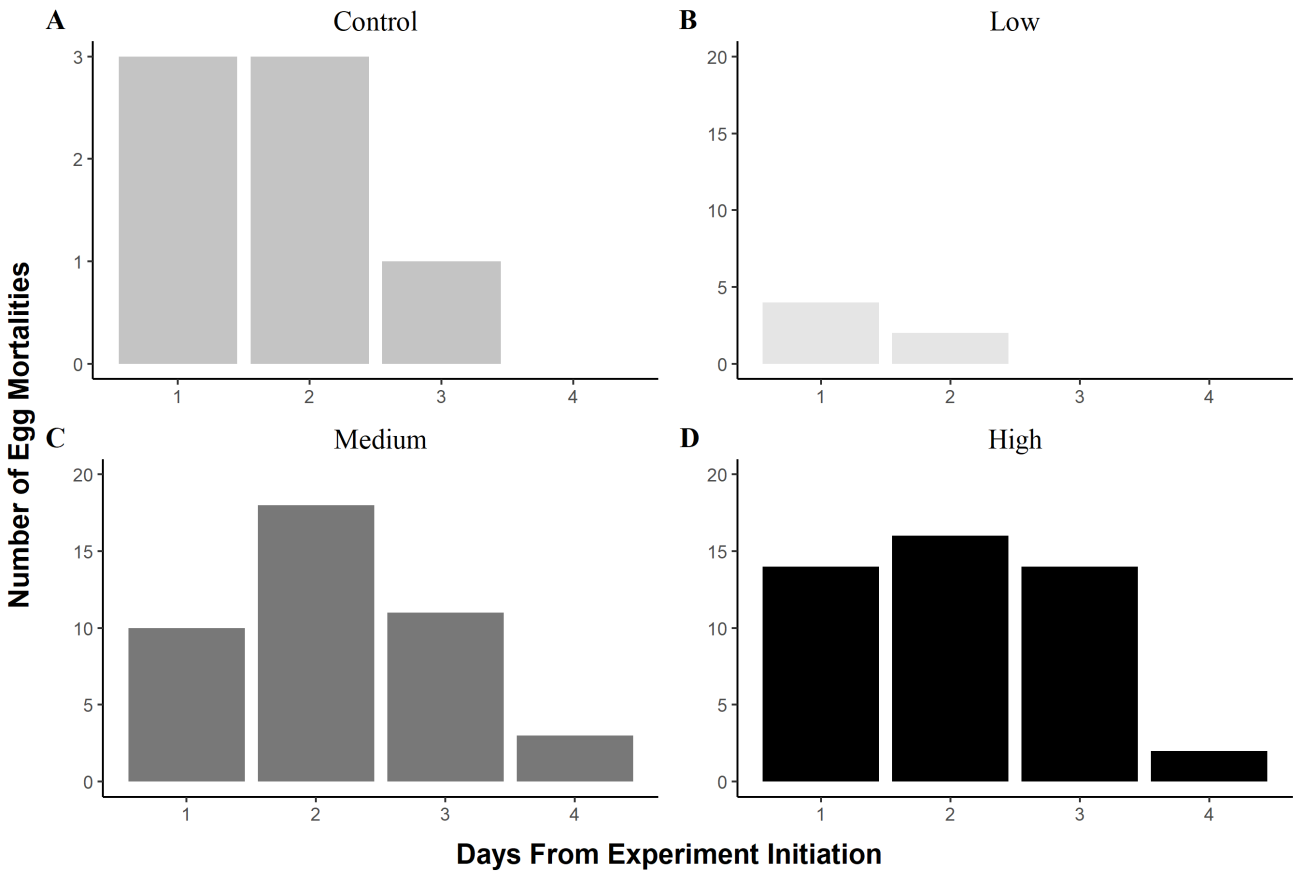


Figure 3(a-d). Number of egg mortalities per day for each treatment in egg-hatching experimentation. Total number of egg mortalities across treatments totaled 101, with the greatest proportion of eggs dying within 48 hours post-fertilization (67%).

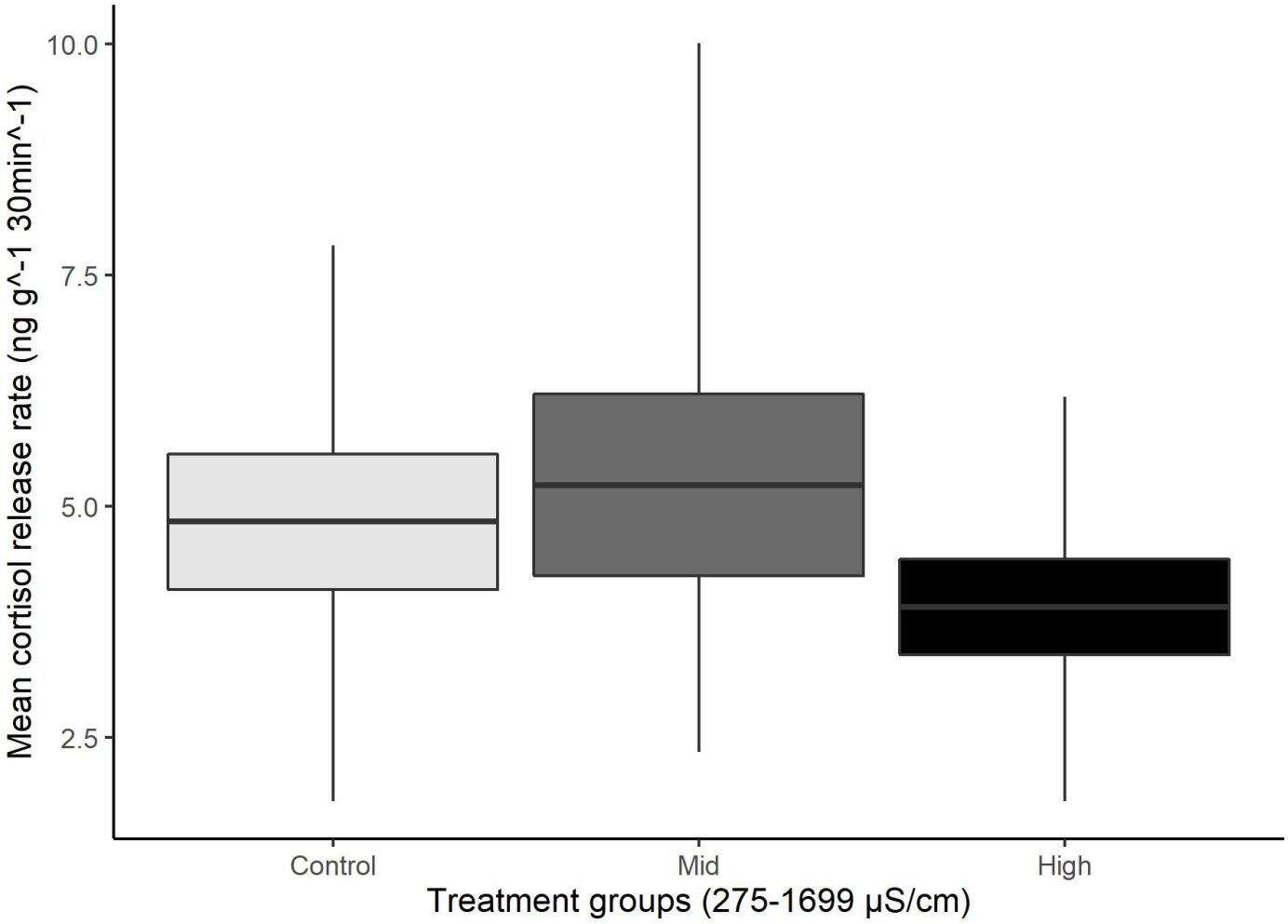


Figure 4. Pooled cortisol levels of adult SRBD in Acute Experiment 1: consecutive day testing, with cortisol values presented as release rate mean ± SE. Overall, cortisol release rates of SRBD were not significantly different among treatment waters (GLMM, EMM; control-moderate, *SE* = 0.907, *p* = 0.60; control-high, *SE* = 0.27, *p* = 0.44; moderate-high, SE= 0.28, p=0.21) and water conductivity alone was not a significant explanatory variable in the amount of cortisol release across the three day experiment (GLMM; *t* = 1.09, *p* = 0.27).

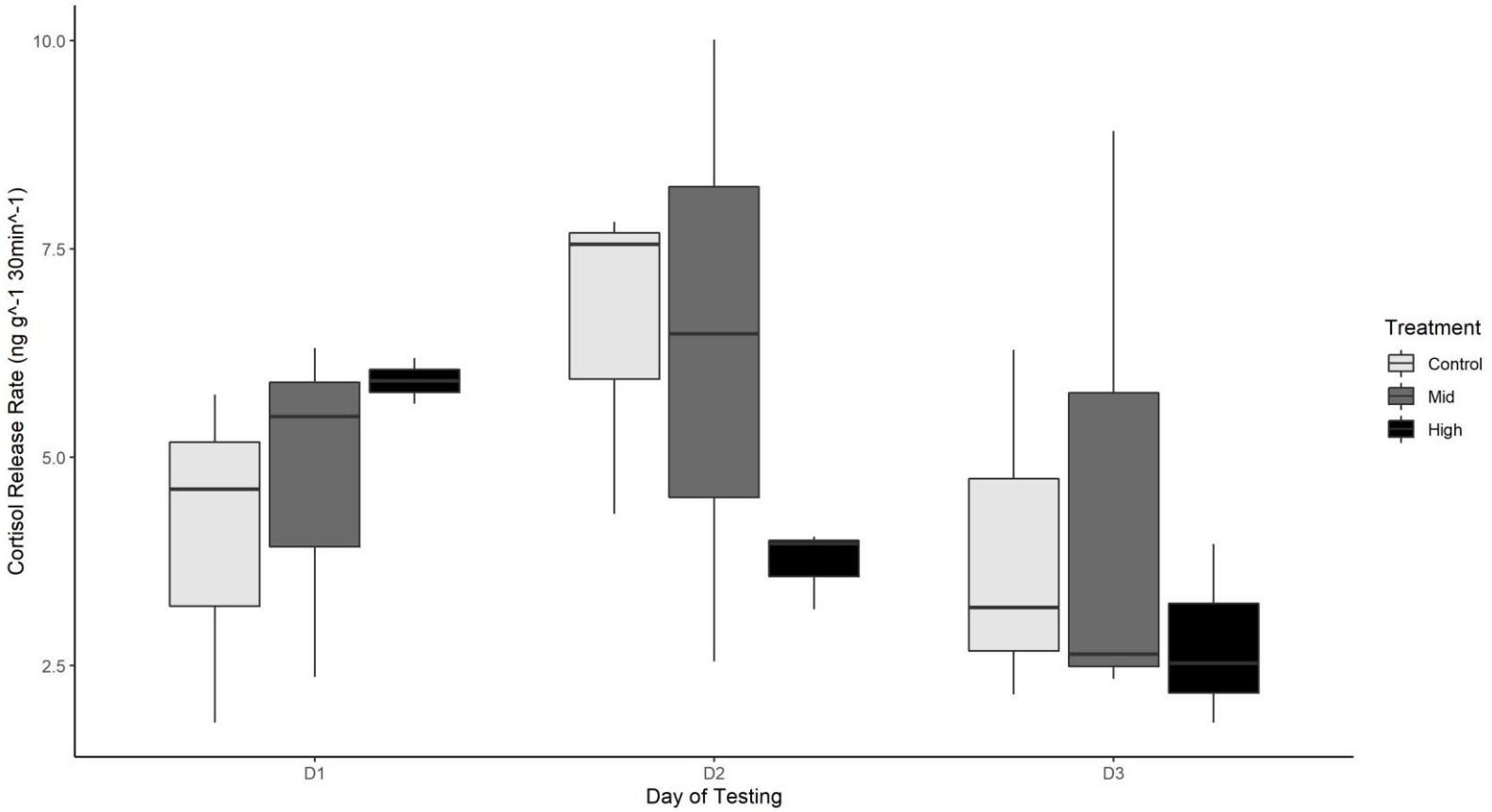


Figure 5. Pooled cortisol release rates from all fish tested in Acute Experiment 1: consecutive day testing, separated by day. Cortisol release rate was significantly influenced by the interaction between the conductivity of treatment water and the day in which groups were tested. On day one, the highest cortisol release rate was among fish exposed to high conductivity water (5.91 ng g-1 30min-1, *SD* = 0.39). Increasing conductivity on days two and three were associated with decreases in cortisol release rate (GLMM; Day 2, t =-2.21, p = 0.027; Day 3, t = -1.28, p =0.173, Figure 6). Controlling for individual variation, our model predicted a 5.91 ng g-1 30min-1 (SE± 1.41) cortisol release rate for fish exposed to high conductivity treatment water on day one, 2.11 ng g-1 30min (SE ± 1.74) for fish exposed high conductivity day two, and on day three cortisol release rate was predicted lowest at 1.99 ng g-1 30min (SE ± 1.8).

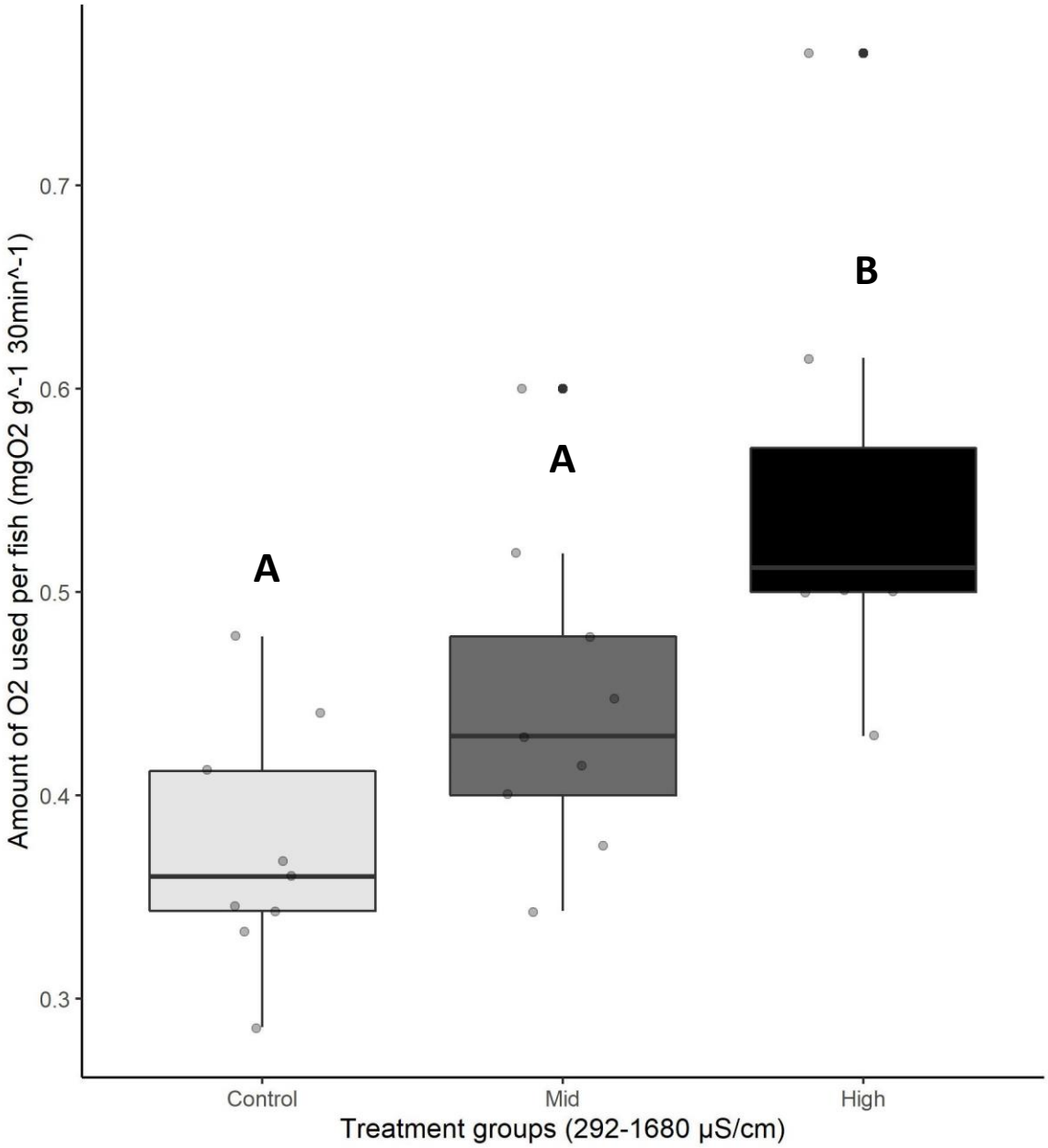


Figure 6. Oxygen consumption of SRBD among treatments pooled from all three days in Acute Experiment 1 (n = 27). Dissolved oxygen concentration was measured before and after every test and was significantly different between control and high treatments as well as mid and high treatments (ANOVA post-hoc Tukey test; control-high, $p < 0.001$; mid-high, $p = 0.028$). Differing letters denote significant differences between groups.

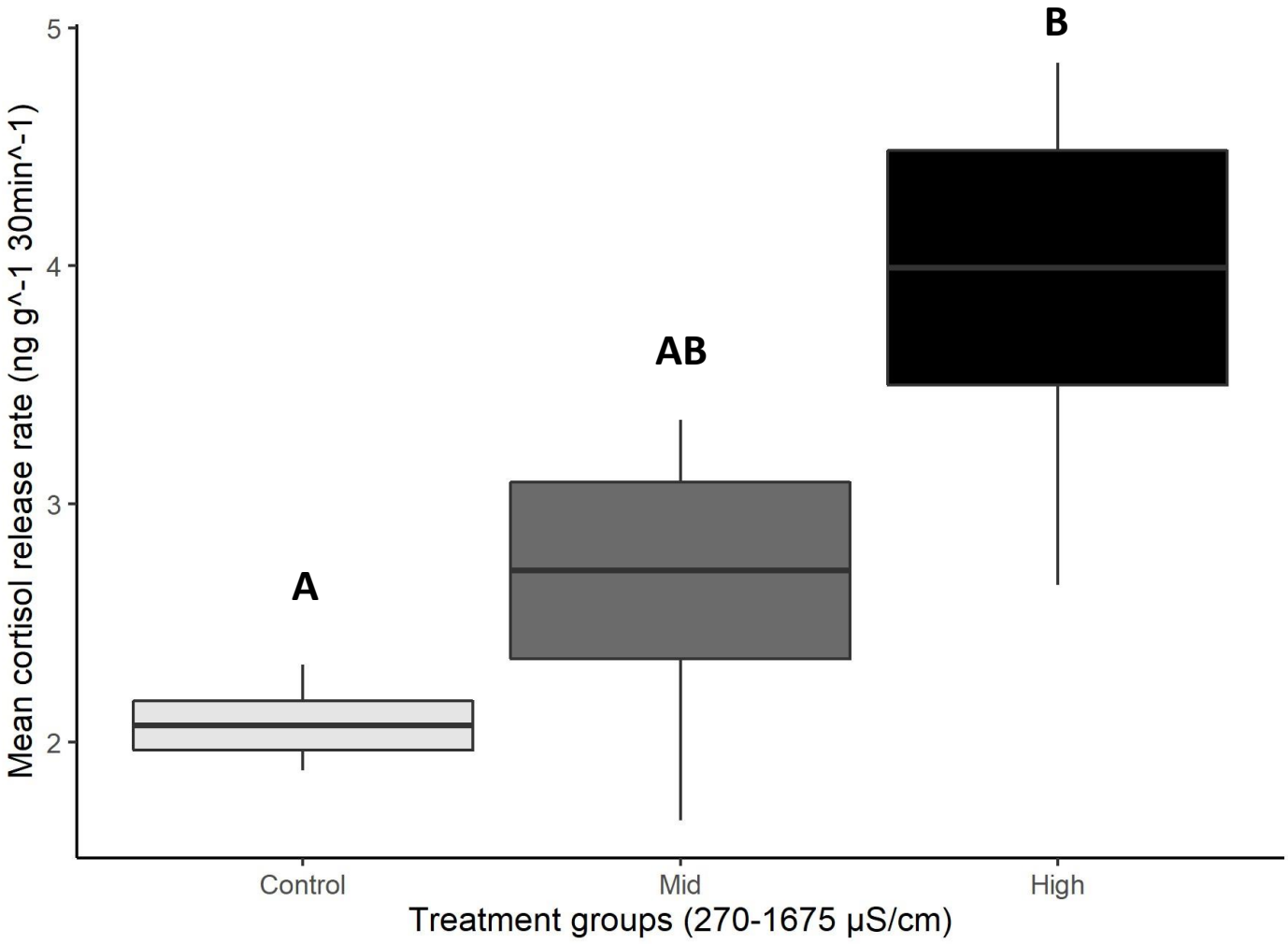
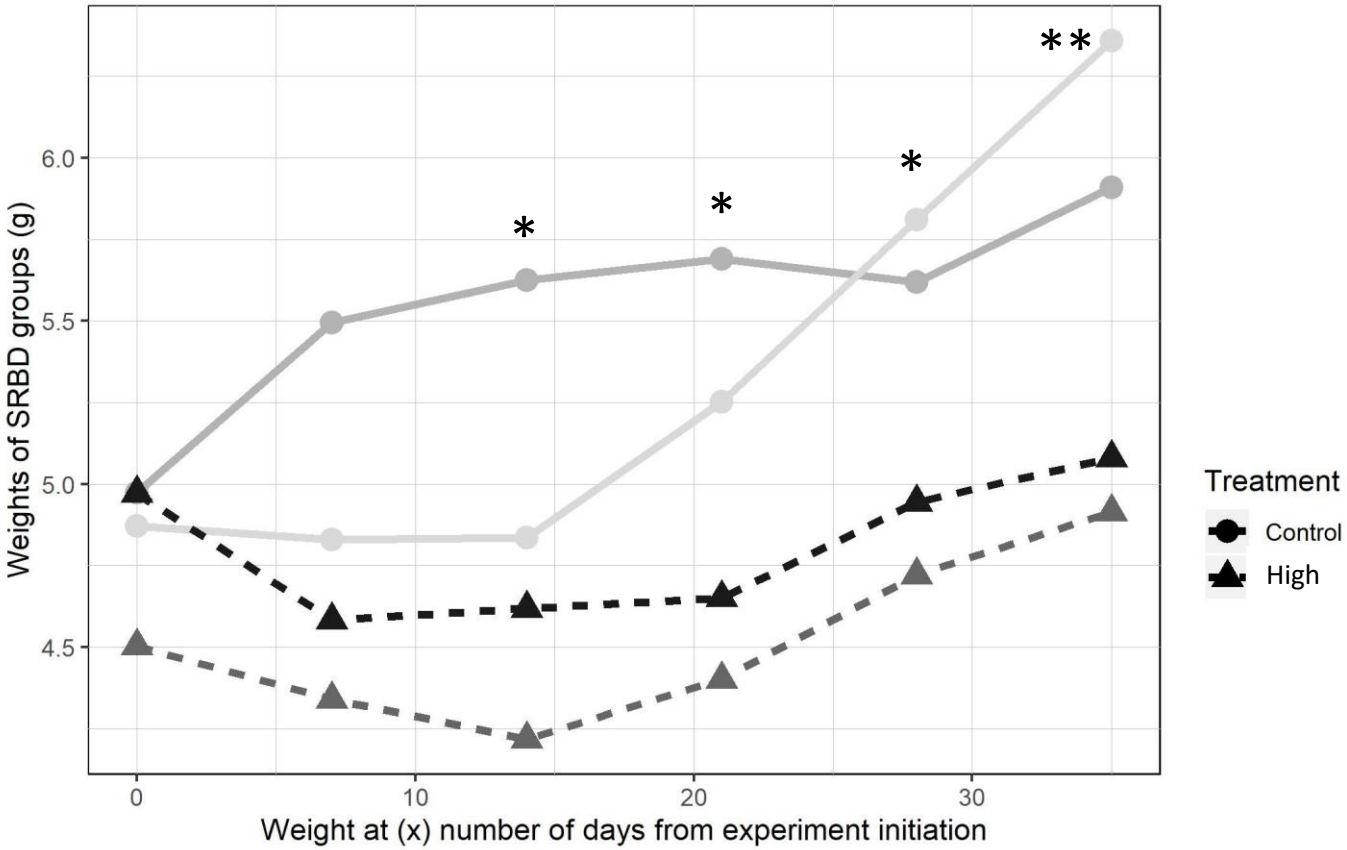


Figure 7. Cortisol release rates (mean ± SE) of SRBD in Acute Experiment 2: single day trial (n = 12). We detected significant differences in cortisol release rate among treatments (ANOVA; $F_{2,9} = 7.34$, $p = 0.013$) and significant differences in cortisol release rate between the control and fish in highly impaired water (Tukey test*; $p = 0.01$*, Figure 14). Differing letters denote significant differences between treatment groups.

Table 5. Water quality characteristics for treatments across Acute Experiments 1 and 2. Specific conductivity averages, with standard deviations, are values derived from individual test beakers immediately prior to exposure. Dissolved oxygen concentrations for Acute Experiment 1 are mean initial measurements and standard deviations of individual test beakers. Dissolved oxygen was not measured in Acute Experiment 2. Other measurements are values measured in batches of treatment water directly prior to experiment initiation.

Treatment	Conductivity (uS/cm)	Temperature (F)	DO (mg/L)	pH	Total Alkalinity (mg/L CaCO3)	Hardness (mg/L as CaCO3)	Sulfate (mg/L)
Acute Experiment 1							
control	281 (6.5)	69.3	7.1 (.07)	8.1	80	119.7	55
mid	845 (10.5)	69.3	7.1 (.09)	8.4	100	307.8	320
high	1686 (13.1)	69.3	7.1 (.09)	8.4	140	718.2	924
Acute Experiment 2							
control	271 (4.5)	69.5	NA	8.1	80	119.7	70
mid	834 (10.2)	69.5	NA	8.3	100	307.8	320
high	1673 (11.5)	69.5	NA	8.4	140	718.2	924



* (p<0.05)
** (p<0.005)

Figure 8. Change in each SRBD group weight over a five-week period (n = 4 groups). Each point represents the total weight of three SRBD individuals per group, and each line represents control (shown in light grey) groups and groups exposed to high conductivity water (shown in dark grey/black). Asterisks represent significantly different weights between treatments at each time point. Treatment type was a significant predictor of weight differences between groups over the course of the experiment (ANOVA; $F_{1,18} = 7.11$, $p = 0.016$, 95% CI: -0.27, -0.04). We found significant differences in weights between treatment and control groups each week from weeks two-five (EMM; week 2, $p = 0.02$; week 3, $p = 0.01$; week 4, $p = 0.01$; week 5, $p = 0.003$). The mean conductivity of control water over the six weeks was 390 µS/cm and mean conductivity of impaired water was 1680 µS/cm.

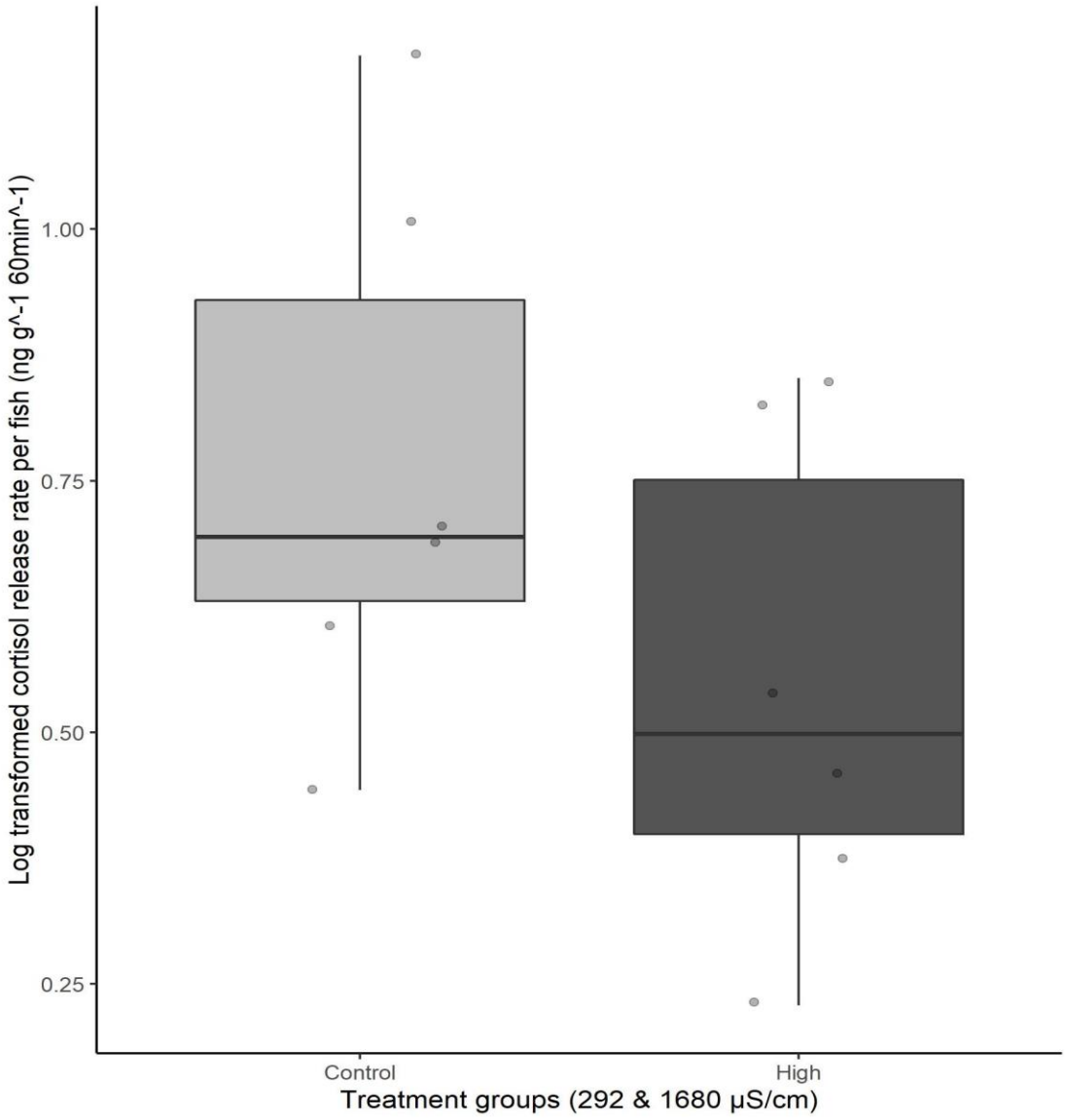


Figure 9. Cortisol release rates of SRBD (*n* =12) after 6 weeks of exposure to control or impaired water. Values reflect 60 minutes of cortisol release after the application of an acute stressor (1 min. net-chasing). We did not detect significance between groups; however, there was a 53% difference in group means (T-test; 95% CI: -0.11, 0.56; $p = 0.16$). The average cortisol release rate for fish tested in control water was 6.96 ng g-1 60 min-1 ($n = 6$, $SD = 4.6$) and the average cortisol release rate of fish after chronic high conductivity exposure was 4.03 ng g-1 60 min-1 ($n = 6$, $SD = 2.28$).

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