Water Pollution Studies

Federal Aid Project F-243-R-22

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Federal Aid in Fish and Wildlife Restoration

Job Progress Report

Colorado Parks & Wildlife

Aquatic Research Section

Fort Collins, Colorado

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The results of the research investigations contained in this report represent work of the authors and may or may not have been implemented as Colorado Parks & Wildlife policy by the Director or the Wildlife Commission.

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State: Colorado Project No. F-243-R22

Project Title: Water Pollution Studies

Period Covered: July 1, 2014 to June 30, 2015

Project Objective: To develop quantitative chemical and toxicological data on the effects of pollutants and water quality on aquatic life, investigate water quality problems in the field, and provide expertise and method development in aquatic chemistry and aquatic ecotoxicology.

STUDY PLAN A: EVALUATING SENSITIVE LIFE STAGES PREVIOUSLY UNSTUDIED

2014-2015 Job A.1. Title: Acute and ultra-acute exposure of fish eggs and sperm to heavy metals during spawning

Job A.1. Objective: Expose egg and sperm of Colorado sport fish to toxicants during the spawning and hardening process to determine if survival or development is impaired.

2014-2015 Job A.2. Title: Acute and ultra-acute exposure of first instar aquatic insects to toxicants

Job A.2. Objective: Whereas aquatic insects are the primary source of food for trout in Colorado, we will expose first instar (one day old) aquatic insects to toxicants and compare sensitivity to exposures at later age classes.

STUDY PLAN B: EMERGING ORGANIC CONTAMINANTS OF CONCERN IN COLORADO

2008-2014 Job B.1. Title: Method development for experiments assessing the effects of organic pollutants such as pharmaceuticals, personal care products and pesticides commonly used in Colorado

Job B.1. Objective: Develop methods for lab or in situ exposures of sport fish and aquatic insects to human pharmaceuticals (e.g. statin drugs), endocrine disrupting chemicals (e.g. estrogen or androgen mimicking toxicants), Permethrin, piperonyl butoxide and/or other insecticides commonly used in Colorado.

STUDY PLAN C: TECHNICAL ASSISTANCE AND COLLABORATIONS

Job C.1. Title: Water quality assistance to Colorado Parks and Wildlife personnel and other state and federal agencies

Job C.1. Objective: To provide technical assistance and expertise, consultation, evaluation and training in aquatic toxicology and aquatic chemistry to Colorado Parks and Wildlife and other state and federal personnel as requested. Assist in the investigation of fish kills. Conduct short or long term experiments to produce toxicity data, or develop site-specific field studies when such data in the literature are lacking or inadequate. Collect and analyze water and/or fish tissues to assess water quality problems. Analyze rotenone in water samples as part of Colorado Parks and Wildlife reclamation projects. Publish results of experiments and water quality investigations in peer-reviewed journals.

STUDY PLAN A: EVALUATING SENSITIVE LIFE STAGES PREVIOUSLY UNSTUDIED

2014-2015 Job A.1. Title: Acute and ultra-acute exposure of fish eggs and sperm to heavy metals during spawning

ACCOMPLISHMENTS

Ultra-Acute exposure of Brown Trout egg and milt to Cu, Cd and Zn during spawning and hardening

Introduction

Early Life Stage (ELS) tests have traditionally been conducted using eggs after spawning and hardening. Previous studies have found that the egg chorion of fish is impermeable to metals (Beattie and Pascoe 1978) and the embryo stage is generally regarded as tolerant to metal exposure (Chapman 1978, Van Leeuwen et al 1985, Brinkman and Hanson 2007). However, most all ELS experiments on fish initiated metal exposure after fertilization and hardening. The spawning and hardening process may be a sensitive life stage. Fish sperm viability in the presence of environmental pollutants might be reduced. During hardening, water and solutes are osmotically pulled into the egg presenting a high likelihood that toxicants present during this 1-3 hour process are directly exposed to embryos. Here we describe method development and experiments attempting to assess risk of exposure during spawning and hardening of Brown Trout (*Salmo trutta*).

Methods

Four year old ripe female and male Brown Trout (*Salmo trutta*) were removed from North Delaney Butte Lake (Walden CO USA) as part of annual CPW spawning operations on Oct 9th, 2014. For each experimental unit two L of water was delivered to plastic thermoses. Thermoses were spiked with appropriate amounts of Cu, Cd, or Zn standards to create 0x, 0.5x, 0.25x, 0.5x, 1x and 3x the acute standards, adjusted for hardness. Each of four experimental blocks consisted of the eggs of one female and the milt of four males. A female's eggs were split into 18 replicates per block using soft silicone infant feeding spoons. Milt was gently stirred and 2.0 ml aliquots were assigned to eggs. Egg and milt was activated using water from thermoses. After two minutes milt was rinsed using water from thermoses and eggs were allowed to harden in thermoses. Eggs were transferred to the Colorado Parks and Wildlife Aquatic Toxicology Laboratory in Fort Collins Colorado. After two hours of hardening, eggs were moved to egg incubation baskets placed under chilled, flow through, water streams (40 ml/min). No additional exposure to metals was assigned after the two hour exposures during spawn and hardening. Temperature settings of chillers were assigned to a temperature equivalent to the mean of all experimental units and were brought from 9°C to 12°C over 24 hours.

Eggs were removed daily if found to be unfertilized (white) or infected with fungus. Mortality, hatch and temperature were recorded daily. Weekly assessment of maturation was recorded with photographs. Fungus was preventatively treated using formalin per Piper et al. 1982. Hatched sac fry were removed and transferred to 2.5 L glass aquaria receiving 40 ml/m flow of dechlorinated municipal tap water. Fry were fed brine shrimp napulii and starter (Size 0) trout chow crumbles until the end of the experiment (30 d post swim up).

Results

Preliminary analysis suggests little statistically significant differences in hatch rates or egg mortality will be found. Natural pickoff of eggs makes discerning treatment effects difficult. Deformities may have been present in higher levels of Cd but these observations were anecdotal. Pickoff of eggs is normal in both hatchery and natural systems. It is difficult to discern metal-related egg mortality given the high background mortality due to natural processes of mating and pathogens. It was unclear if Cu, Zn and Cd was retained in the chorion as implied by Beattie and Pascoe (1978) or if it diffused out with time. Pickoff early in the experiment limited availability of eggs for chemical analysis.

Although the effects of Cu, Cd and Zn exposure were inconclusive in this study, the methods for exposure of egg and sperm cells during mating were proven by this study and will be incorporated during sensitive life stage and full life cycle exposure tests in the years to come. Sublethal endpoints such as pathology, developmental morphology, behavior and swim efficiency will likely be needed to determine if ultra-acute exposure of Cu, Cd and Zn during spawning presents a risk.

Assessment of sperm motility and viability posed numerous problems. Sperm motility and viability in humans is well defined and video analysis software has made sampling far more objective than in years past. Despite these improvements numerous complications were realized while attempting to incorporate sperm motility and viability across numerous treatment levels. To account for parental effects in toxicity tests sperm from the same male should be analyzed after activation in numerous treatment levels of toxicant. In doing so a significant delay exists between the first and last sample in a series. We were unable to account for the decay in sperm viability associated with differences in sperm motility observations over time. Cryogenic freezing and milt extender might negate some of these differences. Method development will be on going.

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2014-2015 Job A.2. Title: Acute and ultra-acute exposure of first instar aquatic insects to toxicants

ACCOMPLISHMENTS

Introduction

Aquatic insects, especially members of Ephemeroptera, are a primary source of food for sport fish. Single species toxicity tests, most conducted with older organisms, have shown aquatic insects to be tolerant to heavy metals but mesocosm and field experiments frequently find insects to be very sensitive to heavy metals (Brinkman and Johnston 2008; Clements et al. 2013). This discrepancy may be explained if early age classes (instars) are shown to be less tolerant.

Members of the genus *Baetis* are a major food for trout. The genera is widely distributed across the world and often comprises the majority of insect abundance in Colorado river systems. *Baetis sp.* are so fundamental to the basic needs of trout that it was once called the "peanut butter of river systems" by Chris Mebane of the US Geological Survey who said "a trout can live on nothing but peanut butter sandwiches [Baetis] and it will be ok, it will live to reproductive age… it will graduate from high school," (North American Benthological Society meeting, Santa Fe, NM, USA. June 9th, 2010). Because of its prime importance to trout species and its wide distribution, *Beatis* nymphs are an ideal test organism for assessing metal toxicity.

Three studies were conducted to determine if tolerance to metals was influenced by body size. The first study utilized unique methodologies and apparatus that enabled a traditional acute toxicity trial to be conducted on first instar nymphs of the genus *Baetis*. The second and third studies measured the body size of insect nymphs from preserved samples of mesocosm experiments exposing communities of insects to heavy metals (Clements 2004, Clements et al. 2013).

Methods

Study 1

Egg masses of aquatic insects were collected from cobble in the Cache la Poudre River, a 4th order mountain stream. Egg masses were examined under a microscope and those likely to be *Baetis sp.* were retained. Each egg mass was assigned to a 25 ml petri dish. Water in petri dishes was replaced daily and was gently aerated. Eggs were incubated at the water temperature observed at collection sites (12-15° C.) Water from the field site was replaced daily and eggs were assessed for hatching organisms twice daily. Immediately upon hatch, organisms were fed a suspension of the diatoms *Navicula sp.* and *Synedra sp.* (Carolina Bio Supply. Burlington, NC, USA)

Fine stainless steel mesh was affixed to the bottom of 24 borosilicate glass tubes (8 mm I.D.) which contained instars during exposure. Glass treatment tubes were mechanically raised and lowered in test tubes (25 x 15mm) every 30 seconds. Continuous-flow serial diluters (Benoit et al. 1982) delivered 40 ml/min dechlorinated municipal tap water (Fort Collins, CO, USA) to test tubes. Test tube racks were submerged in a chilled water bath (12° C). Under a stereo dissecting microscope (Meji EMZ-TR with 20x eyepieces) 15-20 first instars were transferred to exposure tubes using a 100µl pipetter. Organisms were enumerated before and after a 96 hour exposure to Zn at 0, 771, 1710, 3760, 7520, 15040 (22 Oct, 2014), 0, 291, 624, 1390, 3120 (26 Oct 2014) µg/l.

Genetic analysis was used to ensure test organisms were properly identified. Using the statistical package R, raw data were log transformed and the dose.p function in the MASS library was used to calculate LC50 values.

Study II

Archived samples of aquatic insects from mesocosm experiments described by Clements (2004, 2013) were re-examined. In these experiments naturally colonized communities of aquatic insects were exposed to increasing levels of Cu, Cu+Zn and Cu+Zn+Cd mixtures. Head capsules of *Baetis Sp.* and the Heptagenid mayfly *Cinygmula sp.* were measured across all replicates from every treatment level from each experiment. These species were chosen because they were present across most treatment levels and they have head capsules that grow with the age of the organisms. Analysis of age (length) distributions across treatments were examined to test the hypothesis that smaller organisms were selected against in higher treatments.

Study III

Head capsule lengths are not a consistent measure of age or body size for every species of insect. Thus, mass was assessed in every organism of every species in controls from each experiment. Preserved organisms were identified and placed on dry filter paper on a Buchner funnel for 60 seconds and then weighted on an O'Hause GS200D (0.00001 g resolution). Average mass of each taxa was calculated. Slopes of survival data from each taxa published in previous studies (Clements 2004, Clements et al. 2013) were used as measures of susceptibility to toxicants. A weighted regression was then performed to determine if body size was correlated to susceptibility to heavy metal toxicants.

Results

Study I

Preliminary results found mortality at far lower levels than observed using older instars. An LC50 value of 451 µg/L Zn (SD=6.5) was derived. This value is 4% the LC50 (10,020 µg/L) found by Brinkman and Johnston (2008) who exposed late instars of *Baetis tricaudatus* to Zn in the same laboratory under nearly identical conditions.

Study II and III

Statistical analysis of Study II is ongoing. Preliminary results found a significant selection of smaller *Baetis sp.* nymphs in one experiment. Efficacy of using this methodology to determine if larger organisms were more tolerant to metals proved limited. Greater diversity of size classes within a species at the time of exposure would be needed to discern a change in size distribution across treatment levels. Because most aquatic insect lifecycles are synchronous, little diversity of body size was observed in any of the taxa measured.

Analysis of study III is pending.

Discussion

Size or maturity of insect nymphs are rarely reported in aquatic insect toxicity trials. Older more mature organisms are commonly used because they are easy to observe during trials without microscopy and are easier to collect from nature or extract from cultures. A minimal survival requirement of ~95% of individuals in controls is often required for publication or for inclusion in species sensitivity distributions during policy making. This requirement is enforced despite the fact that insects are r-selected species that see natural population loss throughout the life cycle. The minimal survival requirement has perhaps limited publication of studies using sensitive life stages and sensitive species, or perhaps encouraged toxicologists to only experiment with lab tolerant taxa and robust age classes.

Until this study, there have been no methods for assessing acute exposure to small, nearly microscopic, Ephemeropteran insect nymphs aside from use of naturally colonized mesocosms. Such mesocosm experiments often prevent the initial population of organisms from being known. Survival is compared to a subsample of "day zero" communities or is compared to the control. Until recently these have been excluded from derivation of water quality standards despite being far more environmentally realistic than laboratory trials. Mesocosm trials consider predator-prey interactions, ecosystem responses (respiration, productivity), and behavior (emergence, drift) ignored in laboratory trials.

Consultants for stake holders in Colorado have advocated the Colorado Water Quality Control Commission to derive standards for "rudimentary aquatic life" in ephemeral streams. Under the false assumption that ephemeral streams are not used by fish species, the proposed local revisions of water quality standards would consider only aquatic insects and other invertebrates. Historically, laboratory trials using aquatic insects suggest even Ephemeropteran, Plecopteran and Trichopteran species are very tolerant to metals. This study found a marked disparity between the 96 hr aqueous Zn LC50 observed using late instars (10,020 µg/L) and the LC50 for early instars (451 µg/L) of *Baetis sp.* This suggests the belief that aquatic insects are very tolerant to metals could be an artifact of scientists accidentally choosing convenient age classes. Additionally, underprotective standards may result if toxicity experiments on late instar insects are included in the derivation of standards. More effort needs to be made to use only the most sensitive ages when conducting toxicity trials for use in deriving regulatory pollution limits.

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STUDY PLAN B: EMERGING ORGANIC CONTAMINANTS OF CONCERN IN COLORADO

2014-2015 Job B.1. Title: Method development for experiments assessing the effects of organic pollutants such as pharmaceuticals, personal care products and pesticides commonly used in Colorado

ACCOMPLISHMENTS

Analytical methods were developed to analyze benzene by Isocratic High Performance Liquid Chromotography with UV/vis detection using a light frequency of 542nm. This method has proved effective with internal standards and external standards and quality assurance using spiked environmental matrices is ongoing. This method should prove useful for many chemicals that share the properties of benzene, including petrochemicals, insecticides and pharmaceuticals. Piperonyl Butoxide and Permethrin analysis methods were researched but are improbable using instruments available to CPW. However, we have constructed infrastructure that will allow us to perform the extraction of these pesticides at the CPW Aquatic Toxicology Laboratory and outsource analysis to other government laboratories.

Flow-through serial diluters were constructed using only glass, stainless steel and PTFE. These materials minimize adsorption of organic toxicants and all chemicals such as insecticides and petrochemicals. When utilizing flow-through diluters, toxicants may be delivered at a more controlled rate than static or static renewal systems. Methods and infrastructure for culturing daphnia, brine shrimp and lumbriculus in the presence of toxicants were established. This will allow dietary exposure to fish to be included in toxicity trials.

Numerous effluent treatment systems are being tested to allow assessment of pharmaceuticals and petrochemicals. These include carbon filters, hydrophobic resins, and products used to extract fuels in marine industries.

A negative pressure work space was developed that contains treatment cages, chilled bath, food culturing apparati and toxicant diluter. This item was partly donated by BioBUBBLE, Inc. (Fort Collins, CO, USA). This structure allows laboratory staff to safely conduct toxicity trials that involve volatile organics such as benzene, petroleum and solvents.

Toxicology experiments using Gemfibrozil, a statin-like pharmaceutical, were started using fathead minnows as a fish model. With cooperation from the Department of Environmental and Radiological Health Sciences at Colorado State University, this preliminary study is being used to ensure that statins and other cholesterol lowering drugs can be analyzed from aqueous and tissue matrices and that biomedical endpoints associated with statin exposure can be assessed in fish. Following this trial, exposures of longer duration will be conducted and possible synergisms between statins will be examined.

STUDY PLAN C: TECHNICAL ASSISTANCE AND COLLABORATIONS

Job C.1. Title: Water quality assistance to Colorado Parks and Wildlife personnel and other state and federal agencies

ACCOMPLISHMENTS

On-site assessment of rotenone concentrations for reclamation projects in Colorado

Reclamation projects at Parachute Creek (Garfield County, CO, USA) and Zimmerman Lake (Larimer County, CO, USA) utilized rotenone pesticide to remove invasive fish before reintroduction of native fish. Field assessment of rotenone concentrations were needed to ensure target concentrations had been met and ensure rotenone had been neutralized afterwards. The unique analytical capabilities of the Colorado Parks and Wildlife Aquatic Toxicology Laboratory's Mobile Environmental Toxicology and Analytical Laboratory (METAL) were employed to provide this information on site.

Technical support to CPW researchers and the Cooperative Wildlife Research Unit investigating effects of estrogen mimicking pharmaceuticals

Technical support and infrastructure was provided to a CSU and CPW project investigating endocrine disrupting chemicals. The CPW Aquatic Toxicology Laboratory provided space and materials to run an experiment that compared vitellogenin expression of ethinylestradiol exposed fish between wild and laboratory raised fish. This experiment should help to validate and justify the use of vitellogenin as a sensitive endpoint for endocrine disruption in both wild and laboratory raised fish. Ensuring that both laboratory and wild fish express vitellogenin similarly is important for anyone who might want to make direct comparisons between fish from the field and fish from a laboratory. Additionally, space and infrastructure was devoted to rearing common shiners for toxicological assessments of ethinylestradiol.

Technical support to CPW researchers investigating effects of gill lice on sport fish

Technical support and laboratory infrastructure was provided to CPW research investigating gill lice and its effects on sport fish. The Aquatic Toxicology Laboratory at CPW assisted with collaborative gill lice research that is ongoing with the USGS Cooperative Wildlife Research Unit at Colorado State University. Resources supplied included aquaria, life support equipment, and laboratory space.

Technical support to CPW researchers and Colorado State University's investigations of conditioning techniques that promise to increase post-stocking survival of Colorado's sport fish

Technical support and laboratory infrastructure was provided to a joint project between CPW and Colorado State University's Biology Department examining the efficacy of conditioning of hatchery raised fish. Enhancing hatchery environments has the potential to increase post-stocking survival. This project is intended to explore what abiotic environmental factors are important for the development of stocked fish and ultimately survival in natural environments. This project also explores the viability of using environmental enrichment as a means to train hatchery reared fish to recognize local predators and better survive predation events in the wild. In Colorado, it has become apparent that recreational and native fish recovery programs could benefit economically and ecologically from similar approaches to increase post-stocking survival and performance of sport. Aquaria, infrastructure, technical expertise and equipment were donated by the CPW Aquatic Toxicology Laboratory.

Technical support to Colorado State University and Colorado Parks and Wildlife- Investigations of persistant aromatic hydrocarbons in aquatic systems

The CPW aquatic toxicology laboratory provided infrastructure, aquaria, personal protective equipment (PPE) and expertise to efforts investigating effects of diesel fuel and UV light exposure on invertebrate and fish populations. Biomarkers untested in wildlife are being pioneered by Colorado State University using the aquatic toxicology facility at Colorado Parks and Wildlife. Additionally aquatic invertebrates will be exposed to diesel, UV, diesel + UV, and reference conditions in an attempt to determine the importance of UV light to biomarker investigations during a diesel spill scenario.

Technical support to Colorado State University - Investigations of aquatic insect responses to heavy metals and restoration of mine polluted sites

Technical support was provided to Colorado State University Department of Fish, Wildlife and Conservation Biology. Whereas trout populations are directly affected by availability of aquatic macroinvertebrate food sources, CPW provided analytical support and equipment fabrication services to improve assessment of aquatic insect responses to heavy metal pollution. The CPW Aquatic Toxicology Laboratory developed methods for analysis of periphyton health and colonization in mesocosm experiments and field biomonitoring at mine impacted restoration sites near Blackhawk, CO, USA. These results have the potential to predict emergence success in late instar macroinvertebrates.

Technical support to Colorado Parks and Wildlife - Investigations of Common Shiner response to temperature, spawning and culture efficacy

Egg incubation and rearing tanks were provided to CPW managers to assist in method development for spawning and culture of larval common shiner (*Luxilus cornutus*). Experiments looking at growth across a suite of temperature regimes were conducted. Understanding thermal influences on larval *L. cornutus* will lay the foundation for further research, and will provide insight into understanding habitat suitability for this species. Additionally, this study will be useful to federal (USEPA) and state (CDPHE) regulators in creating water quality standards protective of aquatic life. As part of a collaborative effort between CPW Aquatic Research and CPW Platte Basin Aquatics, the CPW Aquatic Toxicology Laboratory has provided the project with experimental design consultations, aquaria, water delivery systems, temperature controllers and fish feed.

Technical support to Colorado State University - Investigations of thermal tolerance

Technical support and equipment was provided to Colorado State University's Biology Department where thermal tolerance of various aquatic organisms is being assessed. These results will aid regulators in efforts to devise protective temperature standards for Colorado.

Technical support to state and federal water quality regulators

Professional opinions and consulting was provided to state and federal agencies (USEPA, CDPHE, CPW) on numerous issues that directly affect sport fish health and habitat, not limited to opinions of state-wide water quality criteria changes, review and comment on site specific alterations to water quality standards and state wide changes to temperature standards. Sport fish populations will only be sustainable if water quality standards are protective of the species and ecosystem functions that sustain these fisheries. Providing advice and counsel to cooperating agencies helps ensure water quality standards will promote sport fish populations in the future.

Technical support to state and federal water quality regulators – Development of temperature standards in areas of multiple stressors

Effects of acute Copper exposure on Critical Thermal Maxima of Mountain Whitefish *Prosopium williamsoni*

Introduction

Anticipated climate changes are expected to have profound effects on structure and function of aquatic ecosystems. Cold-water trout species are especially vulnerable to a warming climate (Ficke et al. 2007). Considerable research has evaluated potential responses of trout species to rising stream temperatures and reduced habitat due to anticipated climate changes (Rahel et al. 2008, Wenger et al. 2011, Roberts et al. 2013). Temperature directly influences distribution of trout species but also interacts with other stressors that may increase risk to populations. Many trout species have shown reduced physical fitness after exposure to sublethal concentrations of Copper (Cu; Buckley et al. 1982, Hansen et al. 1999, Sandahl et al. 2006, and Sandahl et al. 2004). Increasing water temperatures have the potential to increase toxicity of contaminants (Ficke et al. 2007).

Less considered is the possibility that contaminants may adversely affect thermal tolerance and sensitivity to dissolved oxygen. Mountain Whitefish (*Prosopium williamsoni)* can be sensitive to thermal changes and have been found to have lower thermal tolerances than other salmonids (Brinkman et al. 2013, Scott et al. 2015). Laboratory studies were conducted to assess the effects of short term (acute) copper (Cu) exposure on upper thermal tolerance and lower dissolved oxygen (DO) sensitivity of pre swim-up, 30 day post swim-up, and young of year Mountain Whitefish.

Methods and Materials

Study Organisms

The Mountain Whitefish tested as young of year age were spawned in Mad Creek, CO in November of 2013 and reared at the Colorado Parks and Wildlife (CPW) Aquatic Toxicology Laboratory. The fish were held at 5°C (water temperature upon receipt) for thirty days and then acclimated to a holding temperature of 11°C. The Mountain Whitefish tested at pre swim-up and 30 day post swim-up development were spawned in Mad Creek, CO and reared as described above at the CPW Research Hatchery in Bellevue, CO. After swim up, the fish were transferred to the CPW Aquatic Toxicology Laboratory and acclimated from 10°C (water temperature upon receipt) to a holding temperature of 11°C.

Acute Toxicity

Copper toxicity tests were conducted with the three age classes of Mountain Whitefish at 11°C. Water temperature was regulated using chillers that held a water bath at a constant temperature. Toxicity tests were conducted following guidance provided by ASTM method E729 (ASTM 1997). Dechlorinated municipal tap water (Fort Collins, CO, USA) supplied continuous-flow diluters (Benoit et al. 1982) constructed of Teflon, polyethylene and polypropylene components. The exposure levels used in the diluter were based upon experimentally derived median lethal concentrations (LC 50) for unexposed Mountain Whitefish (Brinkman Federal Aid Report 2008). The diluter delivered five exposure levels (1.5 µg/L, 3.0 µg/L, 6.0 µg/L, 12 µg/L, and 24 µg/L) with a 50% dilution ratio and an exposure control. A flow splitter allocated each concentration equally among four replicate exposure chambers at a rate of 40 mL/min each. Exposure solutions were delivered using food-grade vinyl tubing. Exposure chambers were glass tanks with a 9.4 L capacity. Test solutions overflowed from exposure chambers into the temperature regulated water baths. Semi-opaque lids covered the exposure chambers and limited light exposure from dim fluorescent lighting (16h/8h photoperiod) and prevented organisms from escaping. Chemical stock solutions were prepared by dissolving calculated amounts of analytical reagent grade toxicant ($CuSO4 \cdot 5H20$) in deionized water. Chemical stock solutions were delivered to the diluter via peristaltic pump at approximately 2.0 mL/min. New stock solutions were prepared as needed during the toxicity tests. Diluters and toxicant flow rates were monitored daily to ensure proper operation. Biomass loading in test chambers was less than 29% of maximum recommended rates (ASTM 1997). At the start of each 96 hr trial, thirty fry (larval and 30 day post swim-up) or ten fingerlings (young of year) were randomly distributed to each chamber. Fry were fasted for 24 hours before the start of the acute Cu exposure and were not fed during the CTmax or DOmin trials. Mortality data were collected multiple times daily. Mortality was operationally defined as the failure to respond to repeated prodding with a fish net or glass probe. Dead fry were removed from the test chambers, blotted dry with a paper towel, weighed and recorded. Ninety six hour median lethal concentrations (LC50) were estimated using the Trimmed Spearman-Karber technique (Hamilton et al. 1977, Hamilton 1978) with automatic trim. Water quality characteristics of exposure waters were measured at 0, 48 and 96 hours. Alkalinity was determined titrimetrically according to Standard Methods (APHA 1998). Dissolved oxygen, conductivity and pH were measured using electronic meters calibrated prior to each use. Water temperature was taken daily with a calibrated digital thermometer. Water samples for metal analyses were collected weekly from each exposure level with surviving fry. Exposure water was passed through a 0.45 µm filter (Acrodisc), collected in 60 ml HDPE bottles

(Nalgene) and immediately preserved with high purity nitric acid (JT Baker) to pH < 2. Copper, sodium, potassium, calcium and magnesium concentrations were measured using a Thermo Jarrell Ash ICP (IRIS) spectrometer calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard (High Purity Standards, Charleston SC). Water samples for chloride and sulfate analyses were analyzed with a Flow Injection Analyzer (QuikChem 8000, Lachat Instruments, Loveland, CO, USA) using EPA methods 325.1 and 375.4, respectively. Sample splits and spikes were collected at each sampling event. Water samples for dissolved organic carbon (DOC) were gravity-filtered through pre-combusted 47 mm glass fiber filters (1.0 µm size particle retention) (Gelman Sciences Inc., Ann Arbor, MI, USA) using a stainless steel filter holder into pre-cleaned amber glass bottles (VWR Trace Clean) and submitted to a commercial laboratory for analysis.

Critical Thermal Maxima

Critical thermal maxima (CT Max) was measured on fry surviving the toxicity tests using methodology recommended by Becker and Genoway (1979). The CT Max tests were conducted in rectangular glass tanks (18 x 10 x 12 cm). Individual fry were transferred to the tank containing 1.75 L of water at the acclimation temperature. A temperature controller/programmer (B-series Love Controls Division) controlled a submersible 100w Aqueon aquarium heater which heated the water at a rate of 0.3 °C/min. Aeration of the tank maintained saturated dissolved oxygen levels and ensured a homogeneous temperature throughout the tank. Water temperatures were increased until sustained (≥10s) loss of equilibrium (LOE) was observed in the fish being tested. LOE was defined as failure to maintain a dorsal-ventral vertical orientation. This endpoint was used because a fish exhibiting LOE in nature would be unable to escape excessive high temperatures and would most likely perish (Beitinger et al. 2000). Once a fish lost equilibrium, the temperature of the water was recorded and the fish was removed from the experimental apparatus and placed into a 2.5 gallon recovery tank containing water at the acclimation temperature. Each fish was monitored for twenty minutes in order to ensure that it survived the test and regained equilibrium. At the end of the recovery period fish were euthanized using MS-222 and were weighed and measured for total length.

Dissolved Oxygen Minima

Dissolved Oxygen Minima (DO Min) was measured on fry surviving the toxicity tests. The CT Max tests were conducted in rectangular glass tanks (18 x 9 x 12 cm). Nitrogen gas bubbled through fine wood air stones to remove dissolved oxygen at a rate of 1.0L/min. Each tank contained a titanium heat sink circulated with chilled water (5° C) from a head tank to keep the temperature constant in each tank. A stir plate was located under each tank and a one inch stir bar was used on low setting to homogenize the temperature and dissolved oxygen levels in each tank. Individual fry were transferred to the tank containing 2.0 L of oxygen saturated water at the acclimation temperature. After a 30 min acclimation the nitrogen gas was bubbled to displace dissolved oxygen at a rate of two mg/L/hr. Each tank contained a calibrated YSI DO meter probe that took constant % saturation and mg/L DO readings. The testing station contained four tanks that were constantly recorded using digital cameras so that time of LOE and mortality could be observed in the fish being tested. LOE was defined as failure to maintain a dorsal-ventral vertical orientation and mortality was defined as no gill movement with loss of response to stimulus such as a glass probe. Mortality was used as the endpoint in the experiment and was defined as loss of gill pumping or any body movement. At the end of the testing period the fish were weighed and measured for total length.

Results

During the acute Cu exposure no mortalities were recorded in any control or treatment level for young of year Mountain Whitefish. Mortalities occurred in multiple treatment levels during the acute Cu exposure for larval and 30 day post swim-up fish however a concentration of Cu at which half of the test organisms experience mortality (LC 50) could not be established due to lack of mortality in any treatment. All levels of acute Cu exposure were considered sublethal for all three age classes of fish tested.

CT Max testing revealed that the 30 day post swim-up fish had the highest average thermal tolerances out of the three age classes tested after Cu exposure (Figure 2). The results of the CT Max test showed significant differences in temperature tolerances from control organisms for all age classes of Mountain Whitefish tested (Figures 1, 2, and 3). Pre swim-up, 30 day post swim-up, and young of year Mountain Whitefish all had significantly reduced CT Max temperatures at the 24 µg/L treatment level. This was the first significant treatment level for all three age classes.

DO Min testing on the pre swim-up and 30 day post swim-up fish was complicated by the size of the fish and the inability of the cameras to pick up the lack of gill movement indicating mortality. The digital recordings of the DO Min trials did effectively show LOE in the younger age classes however time of death could not be analyzed from the data. DO Min testing on the young of year Mountain Whitefish showed that mortality occurred at a significantly higher dissolved oxygen concentration with fish in the 24 µg/L treatment level (Figure 4).

Discussion

Exposure to sublethal amounts of Cu had significant effects on the CT Max of Mountain Whitefish. An LC 50 concentration was not derived in our acute Cu exposure which could indicate the presence of a greater reduction in thermal maxima at sublethal concentrations above 24 µg/L. Exposure to sublethal amounts of Cu also had significant effects on the DO Min of Mountain Whitefish. These data show that after exposure to 24 µg/L Cu fish were significantly less resistant to lower dissolved oxygen levels in the water. The loss of thermal tolerance after acute exposure to Cu was conserved in all age classes of Mountain Whitefish.

Other resident salmonids of the Rocky Mountain region have exhibited similar responses in CT Max temperatures after exposures to sublethal levels of Cu. Rainbow Trout *Oncorhynchus Mykiss* can be very sensitive to thermal maxima and have shown significant reductions in CT Max at Cu concentrations of 8.0 µg/L (Figure 8; Brinkman unpublished data 2011). Brook Trout *Salvelinus Fontinalis* were not as sensitive as Rainbow Trout however their CT max was significantly reduced at 20 µg/L Cu at two different acclimation temperature levels (Figures 6 & 7; Brinkman 2012). The CT max in Cutthroat Trout *Oncorhynchus Clarkii* has been less affected by acute Cu exposure at sublethal concentrations (Figure 5; Brinkman 2012). The Cutthroat Trout CT Maxima were unaffected below 32 µg/L Cu (Figure 5). These studies suggest thermal tolerance is likely reduced after heavy metal exposure in all Colorado salmonids. When assigning protective water standards regulators should give special considerations to ecosystems where mine pollution and increased temperatures coexist.

Lower dissolved oxygen tolerance and critical thermal maxima are only two metrics of many that contribute to fish fitness. While acute toxicity trials focus primarily on lethality as the primary endpoint, acute exposure to sublethal concentrations of Cu can possibly be affecting many other important traits needed for survival. This shortcoming of public policy is especially obvious in ecosystems where multiple stressors interact.

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Tables and Figures

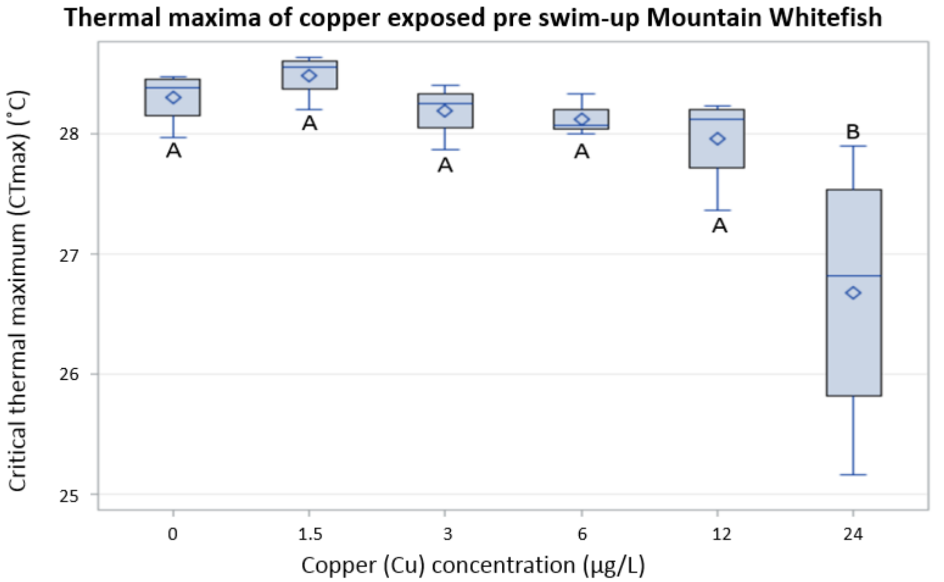

Figure 1. Results of CT Max testing on pre swim-up Mountain Whitefish acutely exposed to sublethal concentrations of Cu.

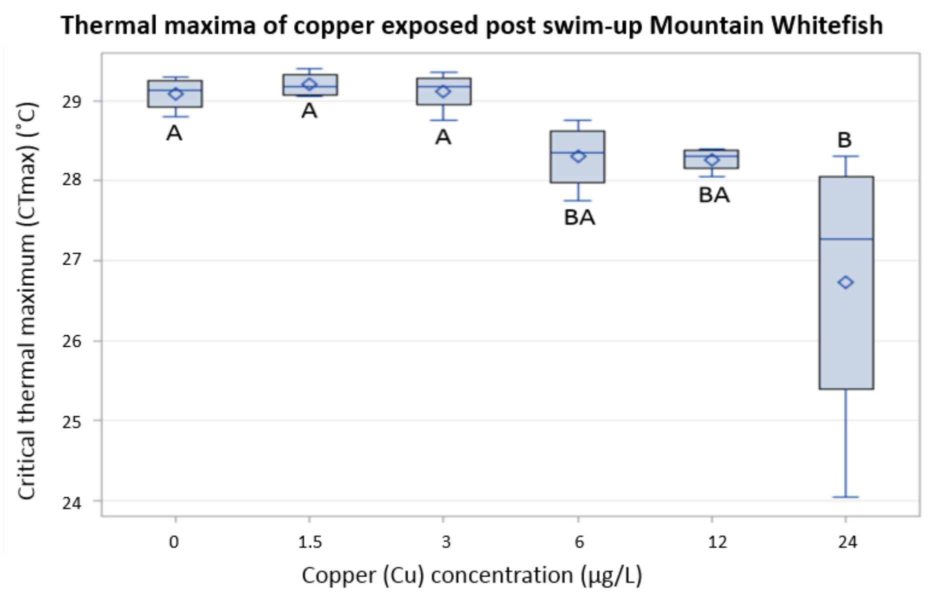

Figure 2. Results of CT Max testing on 30 day post swim-up Mountain Whitefish acutely exposed sublethal concentrations of Cu.

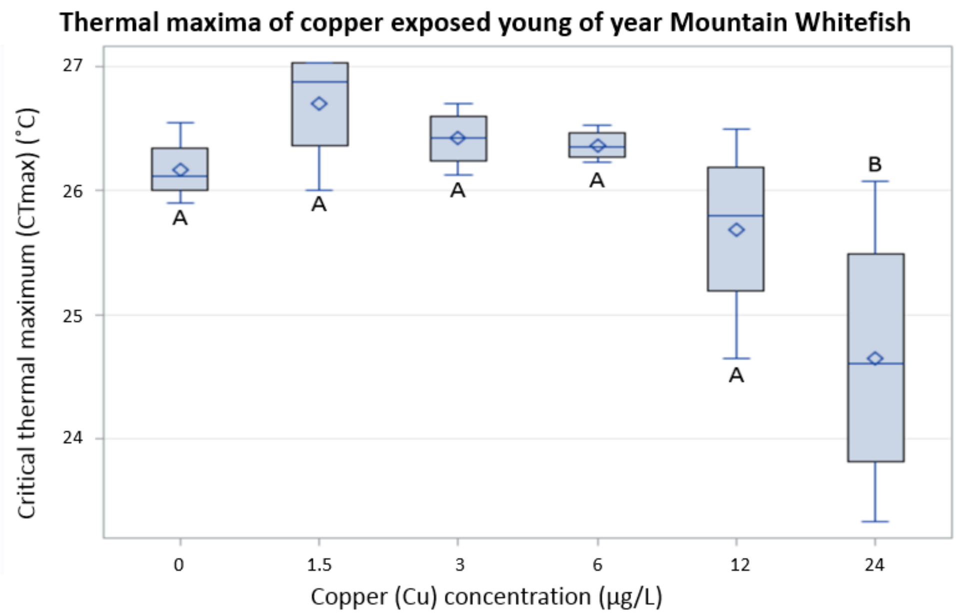

Figure 3. Results of CT Max testing on young of year Mountain Whitefish acutely exposed to sublethal concentrations of Cu.

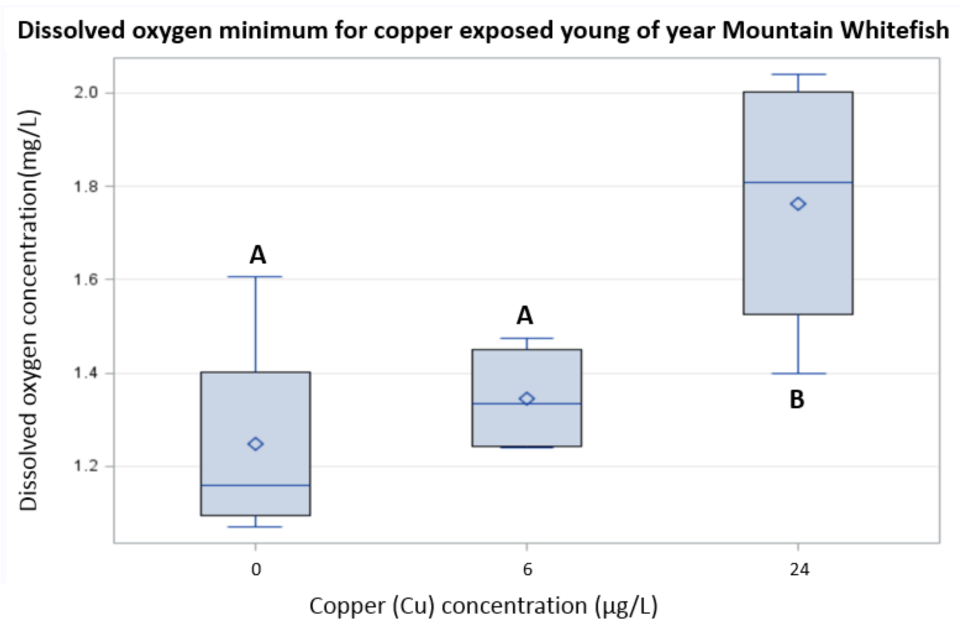

Figure 4. Results of DO Min testing on young of year Mountain Whitefish acutely exposed to sublethal concentrations of Cu.

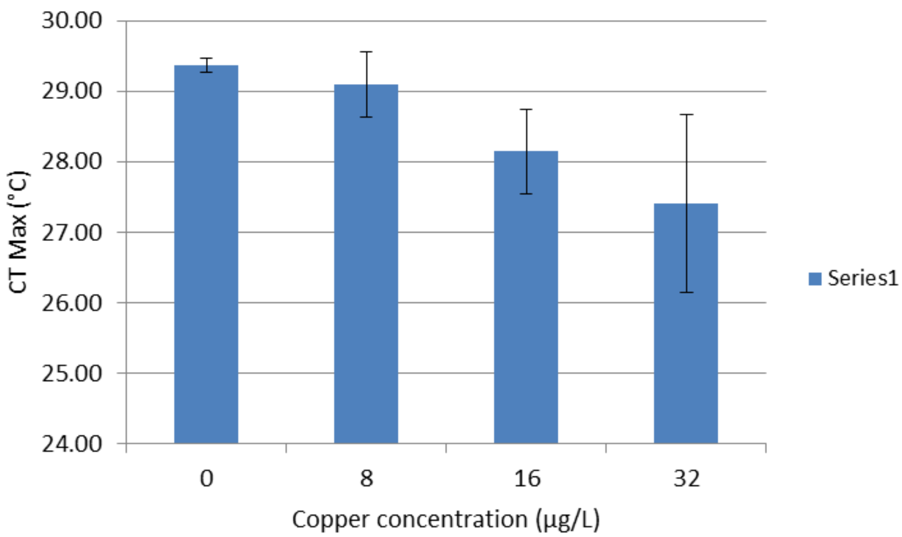

Figure 5. Results of CT Max testing on Cutthroat Trout acutely exposed to sublethal concentrations of Cu (Brinkman Federal Aid Report 2012)

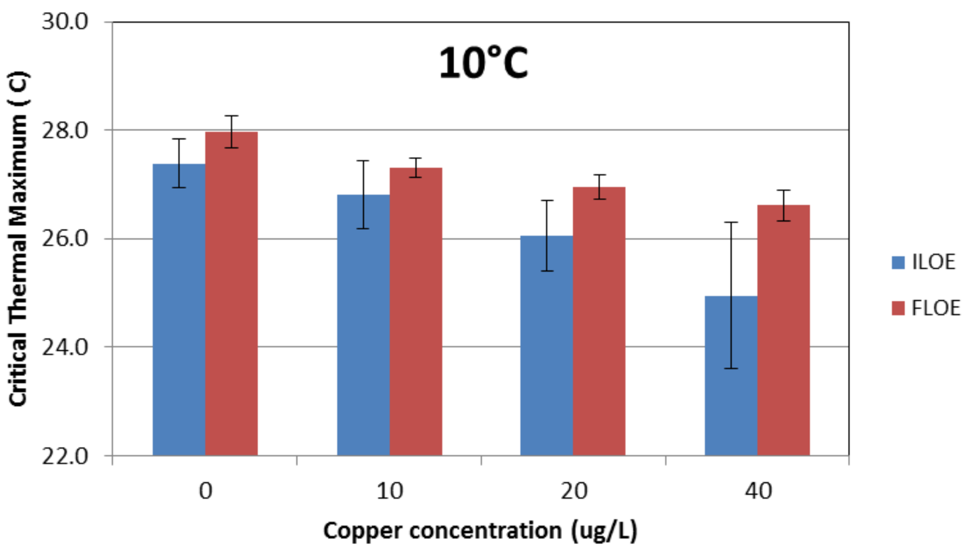

Figure 6. Results of CT Max testing on Brook Trout acutely exposed to sublethal concentrations of Cu at 10°C (Brinkman Federal Aid Report 2012)

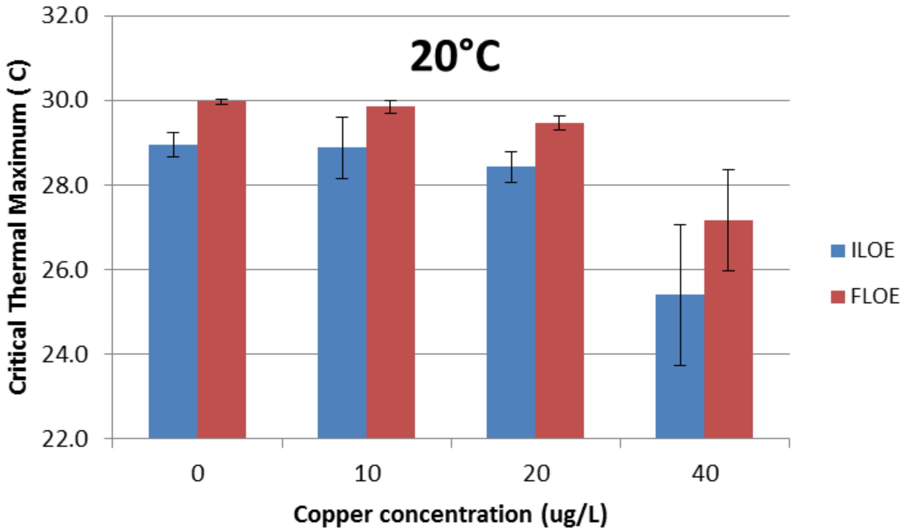

Figure 7. Results of CT Max testing on Brook Trout acutely exposed to sublethal concentrations of Cu at 20°C (Brinkman Federal Aid Report 2012)

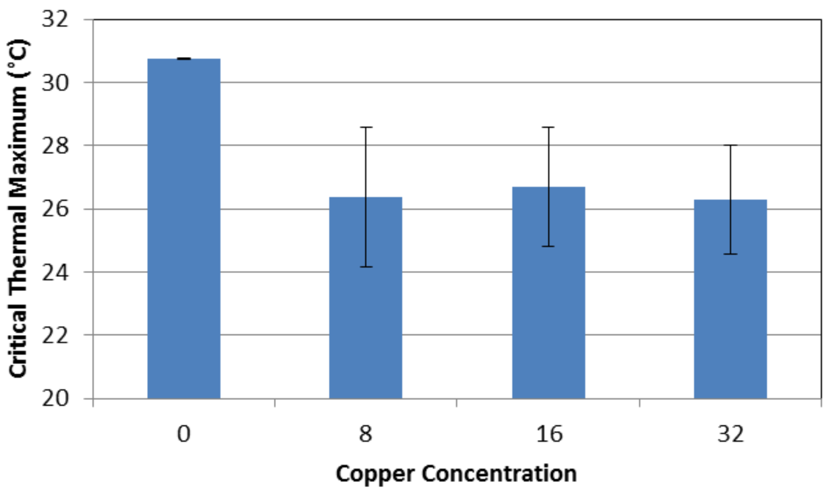

Figure 8. Results of CT Max testing on Rainbow Trout acutely exposed to sublethal concentrations of Cu (Brinkman unpublished data 2011)

Technical support to state and federal water quality regulators and fisheries manager – conduct experiments examining temperature and hydrologic changes associated with climate change

Agitation of Mountain Whitefish eggs induces hatching

Mountain Whitefish has importance as a native Colorado salmonid and as a sport fish. Reasons for population decline are unknown, although climate change, whirling disease and damming and diversion of river flows may all play a role. Agitation, similar to that provided by spring runoff, has an effect on the hatch of European Whitefish. In two experiments, the effect of agitation on Mountain Whitefish is explored. Delayed agitation was shown to promote earlier hatch than consistent agitation. However, when agitation is delayed further, the effect becomes less pronounced. Agitation at any point results in earlier hatch than no agitation. The potential advantages of hatch during spring runoff are discussed, as well as the implications of changes in spring runoff as a result of climate change and dam placement. It is proposed that compromised spring runoff, limiting agitation of Mountain Whitefish eggs is partially responsible for the decline in population.

Introduction

Once the most widely distributed sport fish in the United States, Mountain Whitefish (*Prosopium williamsoni)* had a robust population with a distribution that ranges throughout the mountain northwest of the United States (Behnke 2002). Recent declines in population have been reported in the Big Lost River, Idaho (Gamett et al. 2007) and Madison and Mission Creek, Montana (Vincent 2009). In Colorado, Mountain Whitefish were common in the upper reaches of the Colorado River, the Yampa River and the White River (Schisler 2010), but declines have been found in the Yampa (CPW unpublished data).

The decline of Mountain Whitefish populations could be due to a number of related or compounding factors. The negative effect of higher incubation temperatures on initial survival is well established in salmonids (Elliott and Elliott 2010). Hatch success of Mountain Whitefish eggs is markedly reduced by warm temperatures. Whirling disease co-occurs with the decline in Mountain Whitefish and while whitefish are less susceptible to whirling disease than Rainbow Trout, they are susceptible (Schisler 2010). The presence of dams or diversions has been shown to be disruptive to fish populations (Marmulla 2001). Tail water habitats are less productive than unregulated lotic systems (Paragamian 2002). The physical barrier of a dam, even when mitigated by engineering intended to preserve migratory routes such as fish ladders, is selective within and between species (Marmulla 2001). Regulation of flow can lead to the dewatering of fish eggs (Alexander 2006). Long term effects of dams include a shift from native to nonnative populations of fish (Martinez et al. 1994, Vanicek 1970) in addition to a shift from insectivorous to omnivorous fish (Paragamian 2002). Additionally, dams reduce flows associated with spring runoff.

In the course of rearing Mountain Whitefish agitation has been found to cue hatch in mature embryos (Schisler 2010; personal observations). Previous research suggests European Whitefish eggs (*Coregonus larvaretus*) hatch earlier with agitation (Naesje et al. 1988) and apparently use spring runoff induced agitation as a cue for hatching (Naesje et al. 1986, Naesje et al 1995). It might be advantageous for some insectivorous fish to hatch during spring runoff due to increased availability of food or other favorable conditions. Conversely, previous research has also elucidated the role of mechanical shock on premature hatching. Such early hatching may produce unviable or poorly developed fry with low chances of survival.

Two experiments were carried out to investigate the role of agitation on hatching. In the first, Mountain Whitefish eggs were allowed to develop under three conditions: constant agitation, delayed agitation and quiescence. In the second, Mountain Whitefish eggs were allowed to develop with agitation beginning at differing degree days; resultant fry were analyzed for lipid content as a surrogate measure of health and ability to survive winter and spring conditions.

Methods and Materials

For both experiments, mature Mountain Whitefish were collected either with backpack electrofishing gear in small tributary streams to larger rivers home to robust populations of Mountain Whitefish or through boat electrofishing where tributary spawning aggregations were not obvious. Eggs were stripped and fertilized in the field, placed in 7.5 L water coolers and transported 160 miles (approximately 3.3 hr drive time) to the Colorado Parks and Wildlife Aquatic Toxicology Laboratory in Fort Collins. Upon arrival in the lab, eggs were treated with 1600 ppm formalin for fifteen minutes (Piper et al. 1982). Photoperiod was 12h:12h light:dark.

For experiment 1, forty eggs were randomly distributed to incubation cups within 24 hrs of eye-up (132 degree-days). Incubation cups were constructed by cutting the bottoms off of 355 ml PETE soda bottles, covering the opening with 1000 micron nylon mesh, inverting and suspending in a water bath. Each incubation cup received 60 mls/min flow from a head box containing aerated dechlorinated municipal tap water (Fort Collins, CO, USA) controlled at 6-7°C. Each incubation cup was subjected to one of three treatments (each replicated four times). Quiescent eggs received no agitation. Constant Agitation was started immediately after allocation of eggs to the incubation cups. Eggs in the Delayed Agitation treatment were quiescent for 33 days (approximately 240 degree-days post eye-up) before agitation. Agitation was provided using sufficient aeration from a glass pipette to continually move and maintain the eggs in the water column. Temperature of each incubation cup was measured and recorded daily. Dead or fungus infected eggs were carefully removed using a glass pipette. Hatching was measured four times daily (twice on weekends and holidays). Hatched fry were removed from the incubation cup to facilitate counting. Average degree-days to hatch were compared using ANOVA and treatment means compared using Tukey's HSD.

For experiment 2, eggs were randomly distributed to incubation cups. Incubation cups were constructed from PVC pipes with 1000 micron nylon mesh covering the base. Each incubation cup received 60 mls/min flow from a head box containing aerated dechlorinated municipal tap water (Fort Collins, CO, USA) controlled at 7-8°C. Addition of agitation began five days after spawning and continued at approximately four day intervals up to 17 total additions. Temperature of each incubation cup was measured and recorded daily. Dead or fungus infected eggs were carefully removed using a glass suction tube. Hatching was measured daily. Hatched fry were removed from the incubation cup and frozen for lipid analysis. Lipid analysis was done by Folch extraction (Folch et al. 1957) by Colorado State University.

Results

For experiment 1, temperatures were constant during egg incubation and were consistent among treatments (Table 1). Hatch rate exceeded 95% in all treatments and was not affected by Constant Agitation or Delayed Agitation (Table 10). Mean hatch for eggs in the Quiescent Control occurred near 460 degree-days (Figure 4). Eggs subjected to Constant Agitation hatched near 410 degree-days, significantly earlier than the Quiescent Control. Delayed Agitation led to hatching at approximately 387 degree-days, significantly sooner than both Constant Agitation and Quiescent Control.

For experiment 2, data was consistent with experiment 1. On average, treatments with no agitation hatched later than all other treatments. Those with agitation delayed 30 to 40 days hatched earliest, with treatments agitated before or after 30 to 40 days hatching later.

Analysis of fry fat content showed no relation to time of agitation or hatch (Figure 3 & 4). Mortality, deformations other measures of survival are pending analysis.

Discussion

Agitation has been reported to induce earlier hatching of European Whitefish (*C. lavaretus*; Naesje et al 1988) which is consistent with the observations that spring runoff provides a cue for hatching of European Vendace (*Coregonu albula*) and Whitefish (*C. lavaretus*; Naesje at al. 1986, Naesje at al. 1995).

In experiment 1, agitation of Mountain Whitefish eggs resulted in significantly earlier hatch, suggesting that Mountain Whitefish also use spring runoff as an environmental cue to hatch. Delayed agitation resulted in the earliest hatch of all treatment groups. In experiment 2, addition of agitation at any number of degree days also resulted in earlier hatch. Similar to the delayed agitation treatment in experiment 1, the effect of agitation is most pronounced between 30 and 40 days, at which point eggs hatch earliest. Interestingly, as agitation becomes more delayed, hatch is postponed until comparable with early or near constant agitation. Eggs that experienced no agitation hatched later than all other treatment groups. This further supports use of spring run off as an environmental cue to hatch, but also suggests timing of spring run off is important.

Analysis of fry fat content did not show an obvious correlation with agitation timing. It is possible lipid analysis was compromised by the small size of the samples, or that neither agitation regiment nor hatch time influences fat content. Further studies are needed to establish the effect egg agitation may have on fat content of Mountain Whitefish fry.

Given the importance of spring runoff as a hatching cue (Naesje et al. 1986, 1995) and the results of this study, the existence and timing of spring runoff events might be crucial to the survival of Mountain Whitefish. Newly hatched Mountain Whitefish fry have fully developed mouth parts (Stalnecker et al. 1974) and have been observed consuming food immediately after hatch. Benefits to using spring runoff as a hatch cue may include a more synchronous hatch (Naesje et al. 1995), predatory avoidance advantages and/or greater availability of food sources (Stepanauskas 2000).

This study suggests changes to spring runoff as a result of increasing global temperatures may affect hatch time of Mountain Whitefish. Additionally, rivers that are altered anthropogenically have differing timing, duration and volume of flow from that of natural spring runoff. Alteration of flow regimes via dams or diversions may affect mountain whitefish hatch by affecting temperature and flow, which could negatively affect growth and/or survival of fry. Fry exposed to flows up to 1770m^3/s show only prestress levels of cortisol, and no exhaustive swimming, indicating Mountain Whitefish fry are well adapted to cope with the high flows associated with spring runoff. (Taylor et al. 2012).

Although there are likely many factors contributing to the decline of Mountain Whitefish populations, if we accept agitation in the laboratory as mimicking spring runoff in the wild, these findings underscore the importance of preserving spring runoff by mitigating climate change and limiting dam and diversion disruption of river systems.

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Table 1. Mean temperature (°C) and hatch success (%) of Mountain Whitefish eggs subjected to Constant Agitation, Delayed Agitation and the Quiescent Control. Standard deviations are in parentheses.

	Constant Agitation	Delayed Agitation	Quiescent Control
Temperature (°C)	7.00 (0.27)	7.00 (0.22)	7.04 (0.06)
Hatch Success (%)	99 (1)	99 (1)	96 (4)

Figure 1. Mean time to hatch (degree-days) of Mountain Whitefish eggs subjected to Constant Agitation, Delayed Agitation and a Quiescent Control. Error bars represent standard deviation. Each treatment was significantly different from the others (Tukey's HSD $p<0.05$).

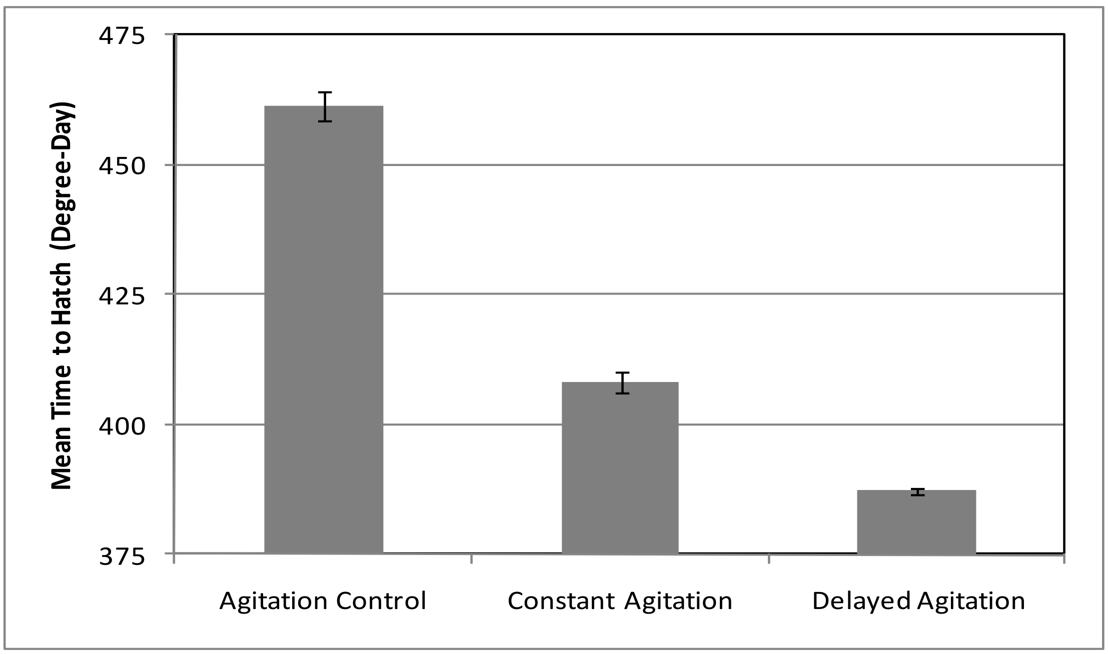

Figure 2. Mean time to hatch (degree-days) of Mountain Whitefish eggs with agitation beginning incrementally.

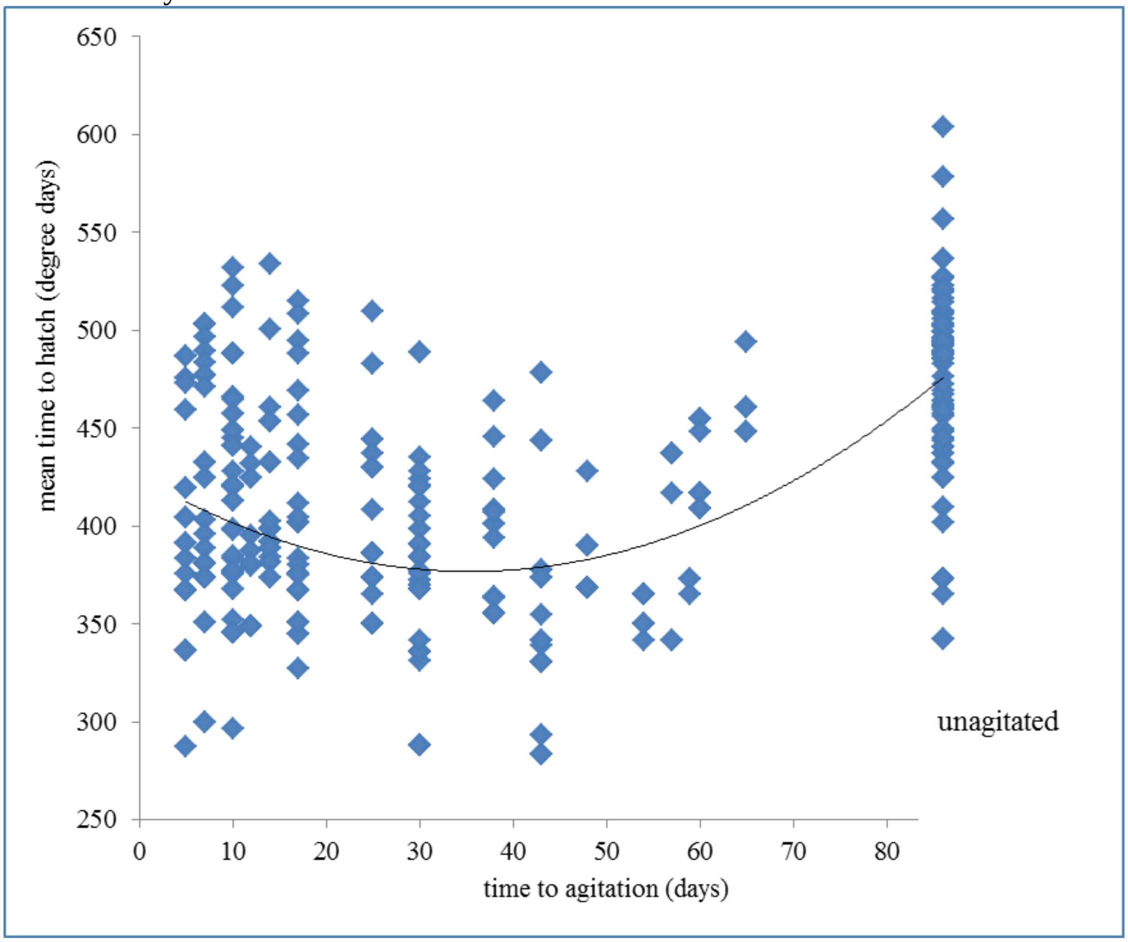

Figure 3. Percent fat in tissue of each composite sample consisting of every live, emerged fry in an experimental unit (incubation basket).

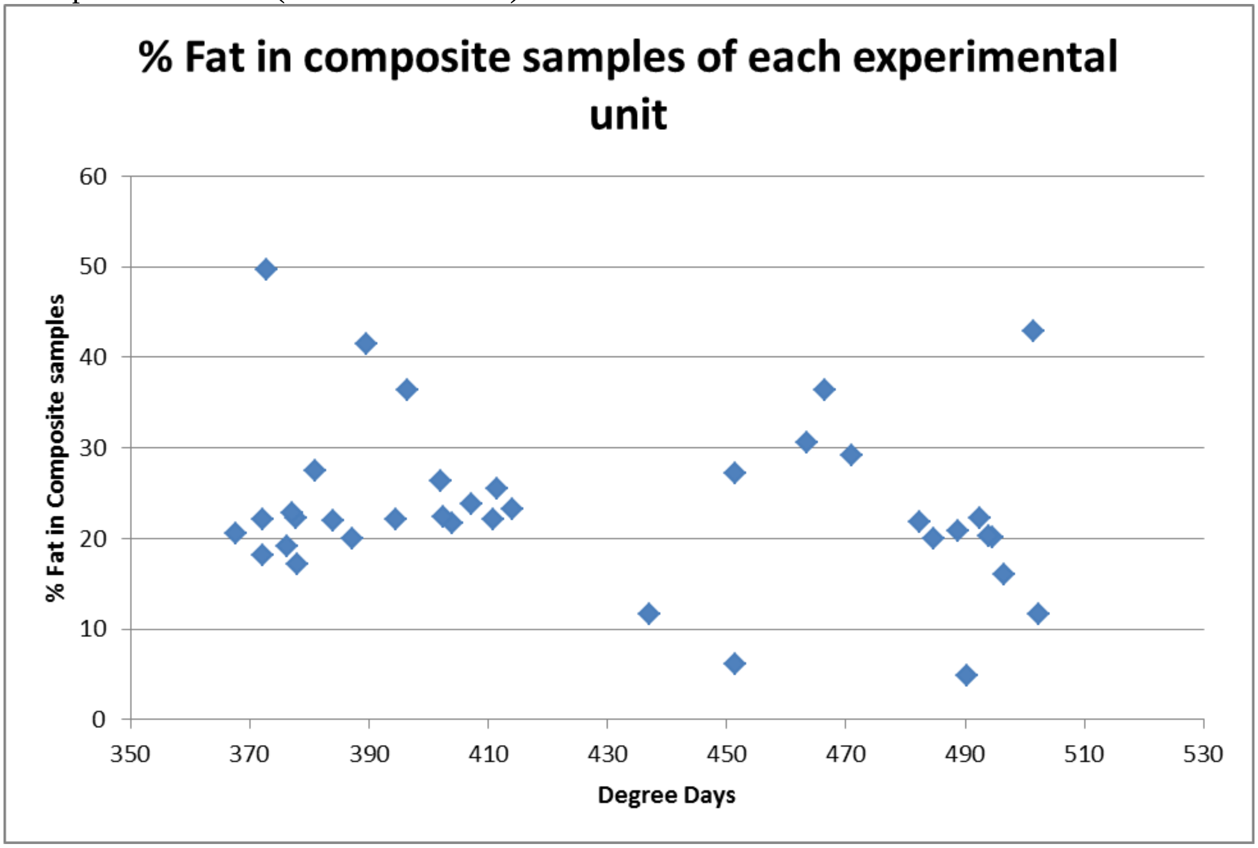

Figure 4. Percent fat in tissue of each composite sample consisting of every live, emerged fry in an experimental unit (incubation basket).