# Water Pollution Studies

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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 and Wildlife policy by the Director or the Wildlife Commission.*

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## NEED AND OBJECTIVE

Prior to mining and westward expansion, Colorado had pristine headwaters supporting dense and mature trout populations. By the 1900s, most of Colorado's headwater rivers could not support fish due to mining pollution. For this reason, Colorado was the first state in the nation to adopt water quality standards to protect aquatic life, preceding the United States Environmental Protection Agency (USEPA) by a decade. The research conducted by the then "Colorado Game and Fish" became the backbone of Colorado surface water standards and later became a majority of data used in numerous national standards in the late 1970s. Additionally, water chemistry assessments and laboratory experiments informed management decisions, determined what age classes could be stocked below mines, and what mine "clean-up" was most needed to improve fisheries. It was Colorado Parks and Wildlife (CPW)'s heavy metal research and Colorado Department of Public Health and Environment (CDPHE)'s regulations that converted rivers deemed "dead" by managers into Gold Medal Trout Streams such as the Animas River (below Durango) and the Arkansas River (between Leadville and Salida). This research and service to managers continues to this day.

Over seven million recognized chemicals exist and 80,000 are in common use (GAO 1994). However, Colorado regulates surface water concentrations of only 63 organic and 15 inorganic chemicals (CDPHE 2013). Colorado's mining heritage has left a majority of watersheds in the Colorado Mineral Belt with elevated metal concentrations. Links between mining activity, metal pollution and degradation of aquatic communities in streams are well established in the literature (Clements et al. 2000). An estimated 20,000-50,000 mines in the western United States produce acid mine drainage (AMD) which seriously affects 5,000-10,000 miles of streams (USDA 1993) and has been described as the greatest water quality problem in the Rocky Mountain region (Mineral Policy Center 1997).

Downstream of urban, industrial or agricultural land uses, organic (carbon based) pollutants have become the predominant and perhaps the least studied form of pollution in Colorado (Daughton 2004). Only a minority of insecticides or herbicides are regulated by standards for aquatic life. Endocrine disrupting chemical classes such as estradiols and pharmaceuticals are known to have an adverse effect on fish populations but the effects of most

of these chemicals are unstudied. For example, statin drugs are marketed to control blood lipids by altering how the body stores and metabolizes fats. These drugs are often highly synergistic and are not completely removed in wastewater treatment. Fat regulation of fish largely affects fish survival and may be altered by exposure to statin pharmaceuticals. Rates of hydrocarbon extraction have increased in Colorado over the last ten years. This presents new risks from extraction and transport processes. Uptake and trophic transfer of hydrocarbons from benthos to fish in both acute and chronic (Lytle and Peckarsky 2001) exposure regimes is well documented (Neff 1979; Giesy et al. 1983; Lamoureux and Clements et al. 1994; Brownawell 1999; Schuler et al. 2003). Increased susceptibility to disease is often correlated with polycyclic aromatic hydrocarbon (PAH) exposure (Damasio et al. 2007; Bravo et al. 2011). Safe concentrations of these chemicals are unknown.

Regulatory agencies such as the USEPA and CDPHE, including the Water Quality Control Commission, act as moderators when building or refining pollution standards. These agencies largely rely on research from external sources and alter standards after solicitations from industry or stakeholders. Colorado Parks and Wildlife is the primary stakeholder advocating for sustainable fisheries in Colorado by producing scientific evidence that ensures water quality standards are protective of fisheries.

Functions of the CPW Aquatic Toxicology Laboratory have historically included:

1- Assess toxicity of emerging contaminants pertinent to Colorado surface waters by conducting toxicity trials on fish, aquatic macroinvertebrates, algae and other fish forage species.

2- Improve state and national water quality standards to ensure they are protective of the aquatic life of Colorado. These standards include toxicants (*e.g.* Fe, Se, Cu, Cd, Zn, Al, Mn, benzene, petrochemicals, pharmaceuticals) and physical properties (*e.g.* total suspended solids, temperature, nutrients). Improved standards rely on improved experimentation that is published in a timely manner and is designed to inform triennial reevaluation of toxicant standards by USEPA and CDPHE. Experiments should:

a) Include rare or sensitive species underrepresented in the literature.
b) When possible, expose rare or sensitive taxa, not laboratory cultured organisms. Expose for environmentally relevant durations, not only standardized 96 hour and 30 day trials. Expose organisms during sensitive life stages (*e.g.* early life stages, egg survival, drift of sac fry, mating, winter survival), consider phenology, species interaction, multi-generational effects, and exposure regimes unique to Colorado.
c) Consider ecologically relevant sublethal endpoints as technology and infrastructure become available to CPW aquatic toxicology laboratory (*e.g.* predator avoidance, olfactory function, fecundity, thermal tolerance, apoptosis, protein carbonyl content, histopathology, blood chemistry).
d) Examine all routes of exposure and all toxic pathways (*e.g.* dietary vs. aqueous exposure, indirect vs. direct toxicity).
e) Increase environmental realism by using natural habitat, natural assemblages, mesocosms, communities, and food chains both in laboratory and field settings.
f) Consider multiple stressors simultaneously, not limited to interactions between numerous toxicants, interactions between toxicants and temperature or interactions between toxicants and disease (*e.g.* whirling disease).

3- Use original research and published research to characterize risk to Colorado's aquatic species. When possible, derive new acute and chronic values for consideration as aquatic life criteria (also known as 'standards' or 'standards for aquatic life'). Employ new techniques to ensure aquatic life standards and management policies are protective of Colorado's aquatic species. Present these findings to regulatory agencies through professional society meetings and peer reviewed publications.

Water quality characteristics and pollution effect fish health and the viability of Colorado's fisheries. Water chemistry and aquatic ecotoxicology demand specialized skill sets

and unique instrumentation/infrastructure. Fisheries managers faced with chronic pollution issues, acute (accidental) spill events, fish kill events and other anthropogenic disturbances often need assistance with analysis of samples and characterization of toxicant effects before, during, and after toxicological disturbance. Site specific and state wide water quality alterations risk compromising fisheries health in Colorado. Decision makers need to be informed of risks to local fisheries. Efforts to restore Colorado's endangered fish species often require precise use of piscicides which are difficult to assess in the field. However, the unique analytical capabilities of the CPW Aquatic Toxicology Laboratory have recently been employed to provide this information on short turnaround using a mobile laboratory. Collaborators at state agencies and universities frequently approach topics that concern CPW's fish and wildlife. By collaborating with these researchers and agencies and by sharing equipment/infrastructure, these projects often produce better data that is more useful to CPW's mission. Technical support conducted by the CPW Aquatic Toxicology Laboratory includes, but is not limited to:

1- 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.

2- Assist in the investigation of fish kills.

3- Conduct short or long term experiments to produce toxicity data, or develop site-specific field studies to address local management decisions or local site-specific variances, when such data in the literature are lacking or inadequate.

4- Collect and analyze water and/or fish tissues to assess water quality problems as requested.

5- Analyze rotenone (and other piscicides) in water samples as part of Colorado Parks and Wildlife reclamation projects.

6- Publish and review results of experiments and water quality investigations in peer-reviewed journals for consideration in policy making by other agencies.

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## OVERVIEW OF JULY 2021 - JUNE 2022 ACTIVITIES

### Research

**Effects of temperature on egg development, hatch success, larval survival, and thermal tolerance of Bluehead Suckers**. Early life stage assessments of Bluehead Suckers (*Catostomus discobolus*) were conducted examining egg development, hatch success, larval survival over a four-month experiment. Thermal tolerance (critical thermal maxima and minima) was assessed on fish acclimated in 8°C, 18°C and 28°C waters. Methods and results are described below.

**Effects of $NaCl_2$ on survival and competition of Mosquitofish and Plains Topminnow.** Research investigating effects of salinity by collaborators Sam Lewis (PhD Candidate) and Dr. Yoichiro Kanno (Associate Professor) of Colorado State University was hosted in the CPW Aquatic Toxicology Laboratory. Salinity is common throughout Colorado, especially in agricultural areas, in drainages where road salt is assigned and in drainages with produced water from coal bed methane operations. The series of studies investigates not only direct effects of salinity but also species interactions between the invasive Mosquitofish (*Gambusia affinis*) and the native Plains Topminnow (*Fundulus sciadicus*) when in the presence of chemical stressors.

**Effects of Road Deicer ($MgCl_2$) on Salmonids.** As a continuation of a series of toxicity trials examining the effects of the road deicer $MgCl_2$ several acute exposures were conducted on Cutthroat Trout (*Oncorhynchus clarkii*). These examined how magnesium chloride and fish density interact to reduce thermal tolerance in juveniles and increase stress hormones. These may inform the loss of salmonid populations near mountain roads such as Straight Creek (Keystone, CO, USA). These studies advanced our use of colorimetric assessments of stress related hormones. Methods and preliminary results are described below.

**Reduced Predator Evasion Following a Sub-Acute (24 hr) Sub-Lethal Diesel Exposure**. As part of a series of experiments characterizing petroleum hydrocarbons, we exposed small-bodied eastern plains fish species to 2.0 mg/L diesel fuel for 24 hours. In non-contaminated conditions we conducted predator avoidance trials in which prey fish from a control group (0 mg/L diesel

fuel) and the exposure group competed to avoid predation. Diesel exposure reduced predator avoidance in all prey-predator species combinations assessed. Methods and preliminary results are described below.

**Research and Development in Rearing and Aquaculture of West Slope T&E species**. Research and development assessing temperature tolerance of these species in late (large bodied) age classes are on-going. This research also includes spawning and rearing of Flannelmouth Sucker (*Catostomus latipinnis)*, Bluehead Sucker and Roundtail Chub (*Gila robusta*) egg and larva.

**Research, Development and Construction of Experimental Streams.** Life support and toxicant exposure systems were fabricated to support lotic (stream and river) species. Temperature, water chemistry, and velocity can be controlled among all sixteen experimental units. These systems will support single-species, multi-species, or mesocosm experiments for future experiments.

**Sample Processing**. Archived insect samples were picked and processed to understand total species composition at various field collected sites in Colorado.

**Effects of Zinc on *Paratanytarsus*.** *Paratanytarsus* sp., a small chironomid ubiquitous across the United States, was exposed to aqueous Zn in a multigenerational experiment. Preliminary results suggest this organism is not protected by state and national standards. Experiments were conducted for 137 and 84 days. Preliminary results from both suggest an EC20 of 11.5 µg/L Zn. Methods and preliminary results are described below.

## Scientific Communication

Effective communication between researchers and fishery managers is essential to promote research studies and address management questions. The objective of the scientific communication page is to provide additional information important for CPW and the Aquatic Toxicology Laboratory through publications, presentations, and research collaborations. CPW Aquatic Toxicology Laboratory staff peer reviewed internal and externally published scientific literature when those papers were pertinent to the unique taxa or unique chemistries of Colorado.

Manuscripts were accepted for publication in peer review journals:


Cadmus, P., R. Friebertshauser, S.F. Brinkman, N. Rhein, and W. H. Clements. *In Press.* Subcellular accumulation and depuration of zinc in periphytic algae during episodic and continuous exposures. Archives of Environmental Contamination and Toxicology

Clift, A. K., A. M. Malmlov, C. L. Wells, P. Cadmus, and P. A. Schaffer. 2022. Branchitis and mortality in Rainbow Trout *Oncorhynchus mykiss* exposed to iron oxidizing bacteria: Diagnostics and management in a Colorado hatchery. Aquaculture, Fish and Fisheries, 2:202– 207. https://doi.org/10.1002/aff2.35

Iwasaki, Y., P. Cadmus, J. F. Ranville, and W. H. Clements. 2022. Stream mesocosm experiments show no protective effects of calcium on copper toxicity to macroinvertebrates. Environmental Toxicology and Chemistry, 41:1304-1310. https://doi.org/10.1002/etc.5308

Kotalik, C. J., Meyer, J. S., Cadmus, P., Ranville, J. F. and Clements, W. H. 2022. Integrated assessment of chemical and biological recovery after diversion and treatment of acid mine drainage in a Rocky Mountain stream. Environmental Toxicology and Chemistry, https://doi.org/10.1002/etc.5515

Riepe, T. B., B. W. Avila, and D. L. Winkelman. *In Press*. Effects of 17α-ethinylestradiol and density on juvenile Fathead Minnow survival and body size. Journal of Aquatic Pollution and Toxicology

Riepe T. B., E. R. Fetherman, B. Neuschwanger, T. Davis, A. Perkins, and D. L. Winkelman. *In press.* Assessment of vertical transmission of *Renibacterium salmoninarum* in hatchery-reared Cutthroat Trout (*Oncorhynchus clarkii*). Journal of Fish Diseases

The following manuscripts are in peer review:

Riepe, T. B., Z. E. Hooley-Underwood, R. E. McDevitt, A. Sralik, and P. Cadmus. *In review.* Effects of temperature on egg development, hatch success, larval survival, and thermal tolerance of Bluehead Suckers (*Catostomus discobolus*).

Riepe T. B., E. R. Fetherman, and D. L. Winkelman. 2022. *In review.* The potential for horizontal transmission of *Renibacterium salmoninarum* among inland trout on a flow-through hatchery unit. Journal of Aquatic Animal Health

Presentations:

*Plenary*: Cadmus, P. The road to Type II errors is paved with great intentions: How efforts to do 'good science' led to bias in both experimental methods and policy. 2002. Meeting of the Rocky Mountain Chapter of the Society of Environmental Toxicology and Chemistry. Denver, Colorado. April 28, 2022.

McConville M., and T. B. Riepe. Water quality testing and hatchery production water sources. Colorado Parks and Wildlife Hatchery Manager's Meeting. Loveland, Colorado. December 14, 2022.

McDevitt R., T. B. Riepe, E. R. Fetherman, and D. L. Winkelman. The effects of *Renibacterium salmoninarum* infection on Brook Trout population characteristics. American Fisheries Society National Meeting. Spokane, Washington. August 25, 2022.

Riepe T. B. Transmission and detection of *Renibacterium salmoninarum* in Colorado inland trout. Department of Fish, Wildlife, and Conservation Biology, Colorado State University Seminar. October 7, 2022.

*Symposium Organizer*: Riepe T. B., B. W. Avila, E. R. Fetherman, and D. L. Winkelman. Enhancing salmonid disease management by understanding pathogen transmission and epidemiology. American Fisheries Society National Meeting. Spokane, Washington. August 25, 2022.

Riepe T. B., E. R. Fetherman, and D. L. Winkelman. Horizontal and vertical transmission of *Renibacterium salmoninarum* in Cutthroat Trout. American Fisheries Society National Meeting. Spokane, Washington. August 25, 2022.

*Special Session Chair*: Cadmus, P., J. Lazorchak, B. Kefford, R. Prosser, D. Pillard. Advantages of using both laboratory and field collected invertebrates in ecotoxicology: challenges and opportunities. Society of Environmental Toxicology and Chemistry. North America 42nd Annual Meeting. Portland, Oregon. November 16, 2021.

## Technical Support

**Technical Support as Needed -** Ecotoxicological support and expertise was provided to CPW managers, Colorado universities, and natural resource management agencies as requested. CPW Aquatic Toxicology Laboratory staff repeatedly provided expert opinions and problem solving for CPW managers and Colorado municipalities concerned about fish health, habitat, and management practices.

**On-site Assessment Of Rotenone -** The CPW Aquatic Toxicology Laboratory conducted on-site assessment of rotenone during chemical reclamation projects to restore native Cutthroat Trout habitat. In addition to in-laboratory Rotenone analysis, CPW's Aquatic Toxicology Laboratory staff provided on-site assessment at the following projects: George-Cornelius Creek Reclamation Project (Red Feather, CO; 2018 to 2022), Rock Creek (Jefferson, CO; 2015 to 2022), Wolf Creek (Wolf Creek Pass, CO; 2021) and Williams Gulch (Red Feather, CO; 2021).

**Milt Extender Production -** Milt extender was produced for federal and state natural resource management agencies across the country during late February and Early March of 2022.

**River Watch Technical Support -** CPW Aquatic Toxicology Laboratory provided advising and support for Colorado River Watch, a non-profit that provides Colorado Parks and Wildlife and other state and federal agencies water quality monitoring and analytical services. Infrastructure improvements to the building were researched, planned and facilitated. Installation of electrical, gas supply, shielding and reaction gas dewars, HVAC, liquid chilling and mass spectrometer related infrastructure improvements were conducted from November 2021 to May 2022. Training and method development on the PerkinElmer Nexion 300 ICP-MS for all staff was conducted in spring 2022 and is ongoing.

**Project Initiation and Method Development of Chemical Assessment of Source and Effluent Waters for all CPW Hatcheries** – To address a long standing need of regular water quality assessments at CPW state hatcheries, T. Riepe and M. McConville developed a sampling program to help CPW hatcheries sample source and effluent waters. Communication with

hatchery staff, including numerous planning meetings, led to the development of user-friendly methods and documentation systems. Sampling kits were delivered to all CPW hatcheries and will soon be monitoring >66 source waters imperative to the production of the state's fisheries. Assessment of metals and nutrients will be included in RiverWatch's production duties.

**Effects of Copper In Low Hardness Waters -** CPW Aquatic Toxicology Laboratory staff helped the University of Alaska-Fairbanks and the Alaska Fisheries Research Co-op Unit build gravity fed serial diluters. This research focuses on a salmon and trout species important to Colorado and examines toxicity in low hardness and low dissolved organic carbon, similar chemistry to Colorado's headwater rivers and reservoirs. Research concluded in early 2022 and equipment was returned to CPW. This work will be published by Drew Porter, Jeffrey Morris, and Benjamin Barst at the University of Alaska-Fairbanks.

**Spill Response -** CPW Aquatic Toxicology Laboratory staff responded to numerous HazMat spills and fish kills. These include some litigation efforts that will not be listed in this report.

**Technical Advising for Water Use and Quality in North Fork of Clear Creek** – P. Cadmus, M. Kondratieff and T. Riepe provided expert opinion, attended field assessments and composed opinions of proposed water diversions, retention and off basin attainment in the North Fork of Clear Creek water basin (Blackhawk, CO, USA).

## On-going Projects – Activities scheduled for the 2022-2023 fiscal year

**Chloride, Sulfate and Ammonia Studies -** Effects of chloride, chlorine, sulfate, nitrate-nitrite and ammonia on a suite of fish, insect and algal species has already begun and is being supported, in part, by Colorado State University (CSU) professors and students. These studies will include dissolved oxygen tolerance, thermal tolerance, competition, chemical avoidance behavior and other endpoints pioneered at the CPW Aquatic Toxicology Laboratory and will make use of the markedly improved detection limits of Colorado River Watch.

**Benthic Macroinvertebrate Sample Identification, Enumeration and Sorting** - Quantitative subsampling, enumeration, and identification of aquatic macroinvertebrate samples from 2015 to 2022 will be completed. These include many from field experiments that simulated flood events before and after the North Fork of Clear Creek (Blackhawk, CO) mine effluent treatment facility was built. Experiments using *Paratanytarsus* sp. are also being processed (enumeration). Use of the Federal Work-Study program has allowed CPW to process samples at significant cost savings while hosting learning experiences for university level students in the biological and chemical sciences.

**Algal Colonization and Competition -** Algal community colonization experiments reported in 2020 will be repeated using molybdenum and/or common herbicides.

**Assessment of Drift and Chemical Avoidance** – Life support and toxicant exposure systems were fabricated to support lotic (stream and river) species. These systems allow single-species, multi-species or mesocosm experiments and can assign temperature, pollution and stream velocity to each of the 16 experimental units. Experiments examining drift of aquatic insects and fish will be conducted seasonally as species are available. Research and development of macroinvertebrate studies was conducted in the spring and summer of 2022. Salmonid exposures planned for the spring of 2023 will likely examine drift response of Cutthroat Trout and

Mountain Whitefish (*Prosopium williamsoni*) at several age classes (sac fry, fry and yearling) to toxicants.

**As Needed Support of CPW Fishery Biologists and Hatcheries** - Continued analytical chemistry support of CPW chemical habitat reclamations will be conducted in late summer and early fall. Milt extender will be delivered to hatchery and biologist staff in late winter of 2022. Research and development in water treatment systems to convert ferrous iron to ferric iron at CPW's Poudre River Fish Hatchery will continue as funding allows.

**Interagency Support and Collaboration** - Continued support of CPW Water Unit goals, CDPHE water quality efforts, River Watch, Colorado universities and researchers.

**CPW Aquatic Toxicology Laboratory Maintenance** - Infrastructure improvements, analytical equipment improvements and improvements to our mobile laboratory will be made as time allows in 2023.

## Effects of temperature on egg development, hatch success, larval survival, and thermal tolerance of Bluehead Suckers (*Catostomus discobolus*)

*T. Riepe*

### Introduction

Water temperature is one of the most important abiotic factors contributing to fish survival. Unfortunately, most fish species (e.g., ectotherms) are poorly adapted to handle variable water temperatures outside of normal annual or seasonal changes (Bruton et al. 2012; Jeffries et al. 2016; Terrazas et al. 2017). Drastic temperature changes can cause physiological and behavioral changes including decreased metabolic rates, ammonia intoxication from the digestive system functioning sub-optimally, and low reproductive success (Brett 1971; Crawshaw 1977; Svobodová 1993). Deleterious effects of colder or warmer thermal regimes often arise due to habitat fragmentation from dams or water diversions. Cold-water pulses below large bottom-release reservoirs and hypolimnetic dams have been well documented (Ptacek et al. 2005; Vanicek 1970) but, increasing drought frequency in the Western US has also exposed fish to abnormally high-water temperatures, and their effects are currently unknown. As lotic systems continue to change, it is important to understand how fish species respond to a broader range and higher variability in both high and low temperatures.

Compounded stressors must also be considered to understand temperature effects on fish. For example, density-dependence and temperature interactions may be experienced as a result of limited habitat during drought and low-flow conditions. During drought and low-flow conditions, a series of stagnant pools can form in streams causing fish to move from low-density to high-density patches, often with higher temperatures than experienced under higher flows. Interacting effects of density and temperature on the survival of larval fish are currently not represented in the literature, although the combined effects have influenced growth of some salmonid species (Bal et al. 2011; Bassar et al. 2016; Bærum et al. 2013; Crozier et al. 2010). However, density-dependence as well as density-independent factors such as temperature, habitat, and water quality have been shown to affect the survival of larval fish independently of

one another (Beverton and Holt 2012; Lorenzen and Enberg 2002). Thus, it is necessary to understand how fish species respond to temperature in the presence of other stressors, such as density, especially because the influence of multiple stressors can be hard to predict.

Critical thermal maxima (CTMax) or minima (CTMin) criteria are commonly used to understand potential thermal tolerance of fish species. Critical thermal limits are measured by constant increasing (CTMax) or decreasing (CTMin) temperature changes from an initial acclimation temperature until loss of equilibrium in the fish is reached, such that locomotive movements are disorganized, and the fish loses the ability to escape from the conditions (Beitinger et al. 2000). Thermal tests do not replicate thermal changes experienced by fish in their natural environment (Brett 1956; Hutchison 1976). Rather, these tests tend to overestimate what the fish can tolerate over a short duration, but they do provide a relevant index to understand how extreme temperature ranges might negatively affect fish (Becker and Genoway 1979; Beitinger et al. 2000; Selong et al. 2001). These measurements are often employed in the creation of water quality standards and determination of impaired habitats. Here, we use this measurement as a relative thermal tolerance for the Bluehead Suckers (*Catostomus discobolus*) evaluated in this study.

The Bluehead Sucker is an integral component of the Colorado River Basin and presently occupies only a fraction of historically occupied waters (e.g. 50% in the Colorado River Basin). Bluehead Suckers are found throughout the upper Colorado River Basin, as well as in the lower Colorado River Basin (limited to the Colorado River and tributaries in the Grand Canyon), and the Weber, Bear, and upper Snake Rivers in Idaho, Utah, and Wyoming (Sublette et al. 1990). Declines of Bluehead Sucker have been observed throughout their native range in Colorado as a result of habitat fragmentation and flow regime modification from dams, diversions, and anthropogenic water use (Bangs et al. 2017; Bezzerides and Bestgen 2002; Martinez et al. 1994; Vanicek 1970; Webber et al. 2012). Lamarra (2007) described thermal niches for Bluehead Sucker eggs, larvae and adults found in the San Juan River in Utah and New Mexico, but water temperatures that induce stress or are lethal have not been identified for life stages in Colorado. Here, we investigated the range of thermal tolerance for larval Bluehead Suckers at three acclimation temperatures and densities. We also determined a temperature appropriate for egg development, hatch success, and larval survival. Our study will directly inform the current and future management of the Bluehead Sucker by anticipating population changes based on the

thermal tolerance and projected water temperature dynamics of the systems in which these fish are found.

## Methods

### *Egg collection and fish rearing*

Adult Bluehead Suckers were obtained by electrofishing from Roubideau and Potter Creeks at their confluence (~ 15 km southwest of Delta, CO, USA) during the 2022 spring spawning season. Two females and six males from Roubideau Creek, and six females and twelve males from Potter Creek, were stripped of gametes and spawned from their respective stream together in a clean, dry container. Immediately following milt extraction, creek water was filtered through a felt bag liquid filter, added to the container, and used to gently agitate the eggs for two minutes to induce fertilization. Eggs were placed into half-gallon insulated water jugs labeled by location, filled with filtered creek water and 400 ppm tannic acid solution to prevent egg clumping, and water hardened for one hour. Following water hardening, insulated jugs were aerated and transported to the Colorado Parks and Wildlife (CPW) Aquatic Toxicology Laboratory (Fort Collins, Colorado, USA). Temperatures in the egg jugs remained at 13.0 $\pm$ 2.9 °C throughout transport.

Upon arrival at the laboratory, the eggs from both creeks were combined and 2,500 eggs were placed into floating Heath stack incubators trays maintained at three different temperatures: 8°C, 18°C and 28°C. Each tray was separated into three, 12.7 x 25.4 cm sections and each section enumerated with approximately 830 eggs. Eggs were treated with a static bath of 1,500 ppb formalin for 15 minutes every two days until eggs were developed (hereafter referred to as eyed eggs) to prevent fungal growth. However, two days after arrival of the eggs, all of the eggs in the 28°C treatment died from fungal growth. Eyed eggs from the other two temperature treatments were counted each day from each section of the trays to determine development success among the two temperature treatments.

Once eggs were eyed in each temperature treatment, 50 eggs were transferred to an egg incubation cup suspended in one of each of the fifteen 18.9-L tanks located in each of the three different temperature-controlled water baths. However, because of the egg mortality that occurred in the 28°C treatment, we used surplus eyed eggs from the 18°C treatment to fill all 15

egg cups in both the 28°C and 18°C treatments. After assigning eggs to egg incubation cups, extra eyed eggs from the 18°C treatment were placed into egg incubation cups in one 75-L tank and held at 18°C for use in CTMax and CTMin trials described below to use if we had any other unexpected egg or larvae loss. Egg cups were made of schedule 40 PVC pipes (65 mm ID cut to 12.7 cm tall) with 0.5 mm mesh floor and received a continuous supply of flow-through water (0.11 liter per min) at the intended temperature treatments of 8°C, 18°C, or 28°C. Each egg cup was hung from the sides of each tank so that eggs sat at a depth of 3 cm. Water temperatures were regulated using chillers and heaters that maintained flow-through water and the water baths containing aquaria at the assigned temperatures. Each flow-through tank and water bath were supplied with dechlorinated municipal water at a flow of 0.11 liter per minute and aerated continuously using atmospheric air pumped through air stones.

Egg cups were checked daily to count egg mortality and hatch success. Once hatched, larval mortality was also counted daily to determine cumulative mortality over time among each tank. After hatching and 50% of the larvae reached swim-up stage, fish were poured from the egg incubation cup into the tank and fed a constant amount of concentrated *Artemia salina* nauplii at 2 mL per day. The *A. salina* nauplii were hatched in a conical hatch tube with 1 g per L of 25 ppt of aerated sea water (Instant Ocean, Blacksburg, Virginia, USA) and incubated for 24 hours.

*Critical thermal maxima and minima*

Critical thermal maxima and minima tests were conducted on all remaining 30 day post swim-up fish from the temperature treatments. Only four fish survived in the 28°C treatment. Therefore, we only used the extra fish from the 75 L tank that were held at 18°C and acclimated the fish to 28°C for 72 hours prior to completing CTMax and CTMin tests. There were no larvae in the 8°C treatment that progressed to the swim-up stage, thus CTMs were not measured. CTMax and CTMin were conducted following recommendations by Becker and Genoway (1979). Our experiment consisted of three densities (low:1 fish, mid:10 fish, high:25 fish) and replicates of 30 trials in the low density, 8 trials in the mid-density for CTMax and CTMin. Unfortunately, we only had enough fish to conduct CTMax (7 trials) and CTMin (7 trials) at high density with the 18°C temperature treatment. Each fish was caught with a net and quickly placed

into individual, rectangular glass tanks (19 x 9 x 12 cm) with 2 L of water held at each acclimation temperature. The same procedure was used to collect fish for each of the three densities (low:1, mid:10, high:25) until the target density was reached.

Temperature controllers (B-series Love Controls Division) were used to regulate the rate of temperature change at $\pm$ 0.3°C/min (18°C/hr) in individual tanks as recommended by Beitinger et al. (2000) with submersible aquarium heaters (for CTMax) or pumps that passed ice water through a radiator (for CTMin). Temperatures in each tank and each trial were recorded with a Traceable Lollipop thermometer. Aeration of the test tanks maintained saturated dissolved oxygen levels and stir bars were used to maintain a homogeneous temperature throughout the tank. Dissolved oxygen was measured before and after each critical thermal trial with a YSI ProODO instrument. Water temperatures increased (CTMax) or decreased (CTMin) until sustained loss of equilibrium (greater than 10 seconds; LOE) was observed. LOE was defined as the failure to maintain a dorsal-ventral orientation and is commonly used as an endpoint for this test (Bennett and Beitinger 1997; Carveth et al. 2006; Selong et al. 2001). In densities greater than one fish, LOE was determined when half of the fish in the tank lost equilibrium. Once the fish lost equilibrium, the temperature of the water was recorded, and the fish were removed and placed into a 5-L recovery tank containing water at the acclimation temperature to determine if they could recover once they were back at the original temperature. At the end of the recovery period, fish were euthanized with tricaine methanolsulfate (MS-222. Western Chemicals Inc., Ferndale, Washington USA).

*Statistical Analysis*

An analysis of variance (ANOVA) was used to determine if there were differences in eyed egg success, hatch success, and larval survival due to the acclimation temperature (8°C, 18°C, or 28°C). Temperature treatment and Heath tray section or tank were used as factors to explain differences in hatch or eyed success or larval survival at the end of the experiment. If there was evidence of a difference in eyed egg success, hatch success, or larval survival, a pairwise comparison with a Tukey's Honest Significant Difference (HSD) adjustment was implemented.

We used an ANOVA to investigate the effects of density and acclimation temperature on thermal tolerance (CTMax and CTMin). Specifically, we sought to determine if there were differences in thermal tolerance when the densities were similar and when densities were different for a temperature treatment. If there was evidence of a difference, a pairwise comparison with Tukey's HSD was used. All analyses were performed in program R (version 4.1.0) and significance was set at 0.05 ($\alpha$).

**Results**

All eggs died at 28°C due to a fungal outbreak two days after collection, and thus were not included in the analysis of eyed egg timing or success. Time to eyed eggs (50% of eggs eyed in a section of the Heath tray) after fertilization decreased with increasing temperature, from 25 days in the 8°C treatment to 8 days in the 18°C treatment ($p < 0.05$, $F_{2,2} = 99.55$). Additionally, eyed egg success differed among the two temperature treatments (Figure A1a). The section of the Heath tray did not affect eyed egg success ($p = 0.23$, $F_{2,2} = 2.14$).

Time to peak hatch (50% of eggs hatching in a tank) from eyed date decreased with increasing temperature, from 8 days in the 8°C treatment to 4 days in the 18°C treatment and 1 day in the 28°C treatment. Hatch success was greater in the warmer temperature treatments ($p < 0.05$, $F_{2,28} = 16.95$) compared to the 8°C treatment but did not differ between the 28°C and 18°C treatments ($p = 0.96$; Figure A1b). Individual tanks did not influence hatch success ($p = 0.50$, $F_{14,28} = 0.98$).

Time to swim-up from peak hatch increased with increasing temperature, from 34 days in the 18°C treatment to 35 days in the 28°C treatment. Larval survival was dependent on temperature, and there were observable differences between the two warmer treatments ($p < 0.05$, $F_{2,28} = 348.15$; Figure A1c). There were no tank effects ($p = 0.53$, $F_{14,28} = 0.94$). Swim-up was not achieved in the 8°C treatment and all fish died after 3 months. Interestingly, fish that did hatch in the 8°C treatment, hatched with little to no yoke sac and the fish with a yoke sac absorbed what was left but never reached swim-up.

Cumulative mortality curves differed for the three temperature treatments (Figure A2). In the 28°C treatment, the total mortality of 99.5 $\pm$ 0.9% (average $\pm$ SD) was reached at 35 days post-hatch, with the first fish dying at 30 days post-hatch. Total mortality in the 18°C treatment was the lowest at 24.0 $\pm$ 15.5%, and was reached at 34 days post-hatch, with the first fish dying at 29 days post-hatch. Total mortality in the 8°C treatment was 99.5 $\pm$ 1.6%, reached at 77 days post-hatch, with the first fish dying at 25 days post-hatch.

*Critical Thermal Maxima and Minima*

Dissolved oxygen remained within 3% of saturation at each acclimation temperature before and after all CTmax and CTmin trails. Only four fish survived in 28°C to 30 days post swim-up and were not included in the trials. Extra fish that were held at 18°C were acclimated to 28°C for 72 hours prior to use in CTMs. Acclimation temperatures in the CTM tanks started at 18.1 $\pm$ 1.6°C for each trial in the 18°C temperature treatment and 27.6 $\pm$ 1.1°C for the 28°C treatments.

At acclimation temperatures of 18°C and 28°C, average CTMax values of the low-density treatments were 32.1 $\pm$ 2.4°C and 33.0 $\pm$ 3.2°C and mid-density treatments were 31.5 $\pm$ 1.5°C and 32.3 $\pm$ 1.5°C, respectively. The CTMax value for the high-density treatment at 18°C was 26.4 $\pm$ 3.3°C. The ANOVA used to investigate the effects of density on thermal tolerance when fish were acclimated to 28°C indicated no difference between the low and mid densities ($p = 0.52$, $F_{1,40} = 0.42$; Figure A3a). However, we did observe a difference among densities in the 18°C acclimation temperature treatments ($p < 0.05$, $F_{2,42} = 15.81$), with Tukey's HSD test showing that high density had a significant effect on the CTMax. When comparing similar densities between the two acclimation temperatures, there were no differences in the low- ($p = 0.196$, $F_{1,62} = 1.71$) or mid-density treatments ($p = 0.3$, $F_{1,14} = 1.16$). Additionally, for the 18°C acclimation temperature, high densities resulted in a 5.9°C decrease in CTMax, whereas when the acclimation temperature was 28°C, CTMax decreased by 0.9°C between the low and mid densities.

At acclimation temperatures of 18°C and 28°C, average CTMin values of the low-density treatments were 7.2 $\pm$ 1.6°C and 15.8 $\pm$ 5.4°C and mid-density treatments were 7.7 $\pm$ 0.9°C and 20.1 $\pm$ 6.2°C at the acclimation temperatures 18°C and 28°C, respectively. The ANOVA used to investigate the effects of density on thermal tolerance indicated no difference in CTMin between the low and mid densities for either fish acclimated to 28°C ($p = 0.29$, $F_{1,30} = 1.187$) or 18°C ($p = 0.32$, $F_{2,42} = 1.17$; Figure A3b). However, when comparing similar densities across acclimation temperatures, the ANOVA revealed differences of CTMin in both low and mid densities between the 28°C and 18°C treatments (low: $p < 0.05$, $F_{1,58} = 68.93$; mid: $p < 0.05$, $F_{1,8} = 44.93$).

**Discussion**

The impact of changing temperatures on different life stages of Bluehead Suckers has received little attention despite their priority conservation status in western Colorado. Bluehead Suckers are long-lived species and typical of such species, higher mortality occurs between hatching and age-one (Ptacek et al. 2005). McAda (1977) indicated that larval survival to age-one was only 0.05. Since this life stage is sensitive to changes, understanding temperature rearing requirements is important for successful management of the species. Spawning and larval rearing often occurs in small perennial and intermittent tributaries with water temperatures between 16-28°C (Bottcher et al. 2013; Fraser et al. 2017; Hooley-Underwood et al. 2019; CPW Internal Reports). Our results indicate that egg development, hatch success, and larval survival are greatest when water temperatures are at 18°C, but constant water temperature during larval rearing is not typical on the western slope of Colorado. Thus, we determined critical thermal ranges for Bluehead Sucker larvae. At 18°C, the range for the fish at a low density was 7.2 $\pm$ 1.6°C to 32.1 $\pm$ 2.4°C. However, when density is high the upper thermal range decreased by 5.9°C. In addition, eyed egg success was greater at the acclimation temperature for 18°C versus 8°C, but after eyeing, all three acclimation temperatures allowed for hatch success greater than 90%.

Warming waters will be a source of chronic environmental stress on the Bluehead Sucker as seen with other fish species as temperatures are increasing over time (Bassar et al. 2016; Ficke et al. 2007). CTMax provides an ecologically relevant index to determine temperature lethality since wild fish that encounter a temperature outside of their threshold are not likely to escape from predators or move to a cooler location. We found no reports of CTMax values for Bluehead Sucker at various acclimation temperatures. Thus, our values of 31.5 $\pm$ 1.5°C and 33.0 $\pm$ 3.2°C, at acclimation temperatures of 18°C and 28°C, respectively, represent the only CTMax data available for this species. Although we did not find a difference in CTMax between the two acclimation temperatures, density had an influence on the CTMax with the 18°C acclimation treatment.

Many of the small perennial and intermittent tributaries used for spawning are heavily diverted for agricultural and other human uses in Colorado. Low flow and elevated temperature conditions are common and may become stressing or lethal to Bluehead Sucker and other fishes present. For example, in the dry summer of 2018, irrigation and municipal water diversion from the San Miguel River in southwest Colorado resulted in a near complete dewatering of 8.5 km of river and a large fish kill that included over 4,000 Bluehead Suckers, and high temperatures were cited as the probable cause of mortality (CPW Internal Reports). During drought conditions, water levels and volume in intermittent or perennial streams can drastically decrease in days or even hours, leaving a series of diminishing pools. The pools may succumb to fluctuating temperatures daily. For instance, temperature changes in a matter of hours have been documented from 10°C to 28°C, which is equivalent to an increase of 0.1°C/min (CPW Internal Reports). This increase is smaller than what we used in our study (0.3°C/min) but we do believe that stagnant pools may increase at the rate we used or higher. If the temperature increase is occurring after Bluehead Suckers have hatched, our results indicate that they will not survive in temperatures near 28°C. In addition, low flow days may trap the larvae in the stagnant pools. Thus, the unavailable habitat and increased water temperature in the pools may act as a density-dependent bottleneck that will limit the survival of larval Bluehead Suckers.

Similar to Bluehead Sucker CTMax, we did not find any published reports of CTMin. Our study indicates that at an acclimation temperature of 18°C the CTMin is 7.2 $\pm$ 1.6°C and at

28°C is 15.8 ± 5.4°C. Acclimation temperature affected the CTMin resulting in a reduced thermal tolerance at higher temperatures. Larval Bluehead Suckers typically drift downstream after emerging from the egg and our results indicate that when larvae are in warmer water initially, they are unlikely to tolerate a cold pulse greater than a 12.2°C decrease. This is important to note when rivers are regulated by bottom-release dams. Bluehead Sucker and other sucker larvae are typically produced in abundance in Escalante Creek, CO which is a small tributary to the Gunnison River and is a dam-controlled water body. On one day of the 2022 larval drift period (June 10$^{th}$), peak temperatures in Escalante Creek and the Gunnison River below the confluence were recorded at 27.1°C 19.2°C, respectively. Larvae drifting between these two locations experienced at least a 7.9°C decrease in water temperature due to the cold water coming from the dam. Our CTMin test suggests that many fish can tolerate this change, but not all fish can, which will influence overall recruitment. Other stressors such as decreased dissolved oxygen, low flow, water chemistry, or fluctuating temperatures may decrease the tolerance of the fish as we observed with density in the 18°C treatment. Therefore, we suggest further research to address the effects of multiple stressors on thermal tolerance of larval Bluehead Suckers.

Our study indicates that hatching success can occur at all three acclimation temperatures, but the temperature of 28°C will negatively affect the proportion of eyed eggs. However, our conclusions of eyed egg success in the 28°C treatment was limited because of fungal growth experienced two days after fertilization. If this did not occur, eggs may have developed at 28°C since we have noted adult Bluehead Suckers spawning near this temperature in Colorado (CPW Internal Records). Flow also allows for the decreased probability of a fungal growth, and we may have not had the appropriate flows to prevent the growth. However, in the wild, low-flow periods increase temperature and the amount of flow directly passing over eggs, which could cause increased fungal rates. Interestingly, hatch success was greater than 90% among all three acclimation temperatures. However, we did note variable times from egg development to hatching between temperatures. Specifically, eggs at 8°C took 8 days to hatch, which is different than the 1-4 days in the 18 or 28°C treatments. During the hatch period we also noted that many of the fry did not hatch with a yolk sac which may affect recruitment in wild populations. Other studies suggest colder temperatures limit recruitment due to extended time in sensitive, larval

stages (Rice et al. 1987), but our results may suggest that larval survival can also be low at cold temperatures due to limited available nutrients. However, other explanations that disrupt the phenology of egg development including an agitation cue that is timed with runoff, timing with optimal foraging and navigable flow rates, and slow warming temperatures in the spring and summer may also affect larval survival in the wild (Martin 1999; Warkentin 2007). The 28°C temperature also induced near 100% mortality indicating that larvae cannot tolerate high temperature for long periods and 18°C is the most appropriate temperature for survival in our study. Future studies should investigate what affects larval survival at various temperatures and thereby recruitment of the Bluehead Sucker.

## Conclusions

We used an experimental approach to assess the impact of variable water temperatures on early life stages of Bluehead Sucker, a declining species of the western US. These fish typically experience variable water temperatures in their spawning and migration habitats ranging from 10-28°C. Our study indicates that the acclimation temperature of 18°C resulted in the highest egg development, hatch success, and larval survival. Based on their physiological responses to the CTMax and CTMin tests, Bluehead Sucker may be more susceptible to colder temperature changes if they are acclimated to higher temperatures. Therefore, if the larvae are present when cold pulses of water from bottom-release dams occur, or if they drift into such waters, they are not likely to survive if they cannot retreat from the area. Although upper thermal temperature ranges did not differ between 18°C or 28°C acclimated fish, density of the fish does seem to influence the upper thermal limits at 18°C. Larval Bluehead Sucker often aggregate in pools during low flow conditions and if they cannot retreat from warming areas, periods of increased water temperatures may be detrimental to survival. Understanding the thermal regime for this threatened species is crucial to effectively manage the Bluehead Sucker.

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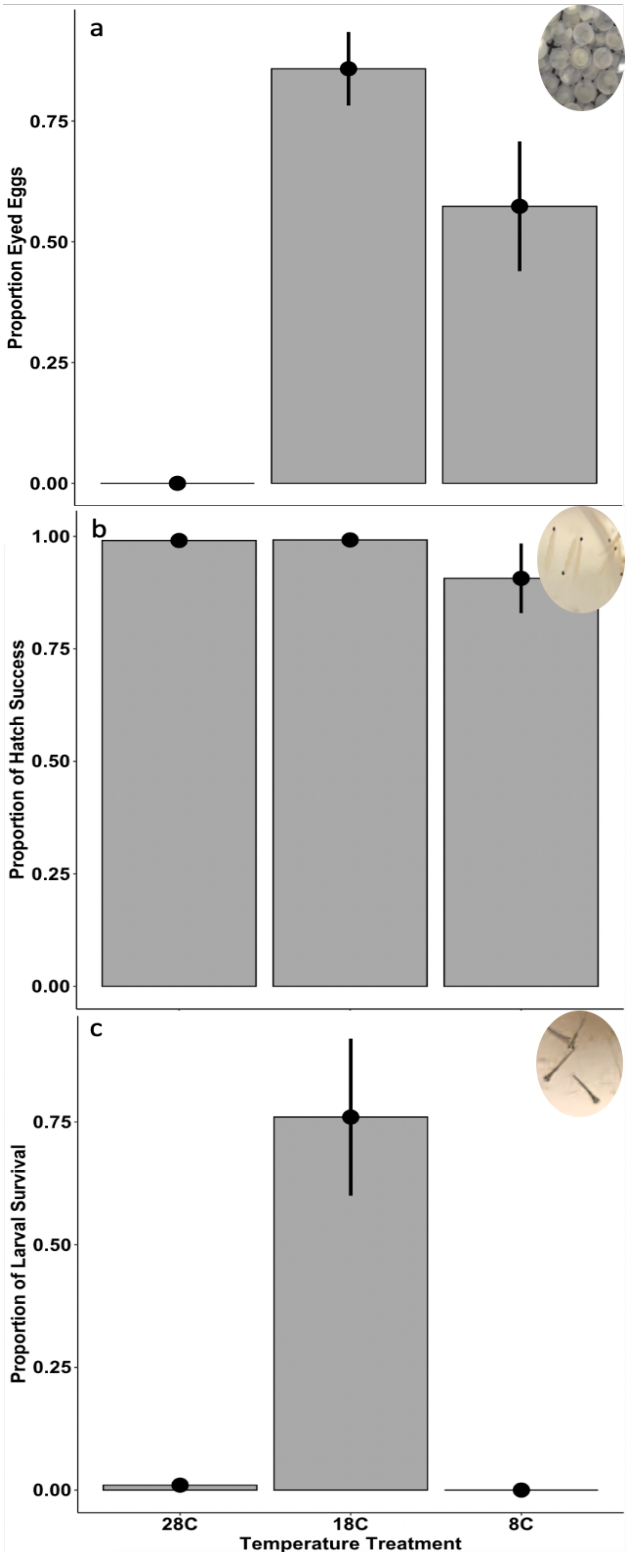


**Figure A1**. A) Eyed egg success, b) hatch success, and c) larval survival for three acclimation temperatures (28°C, 18°C, 8°C). Vertical bars represent the standard deviation.

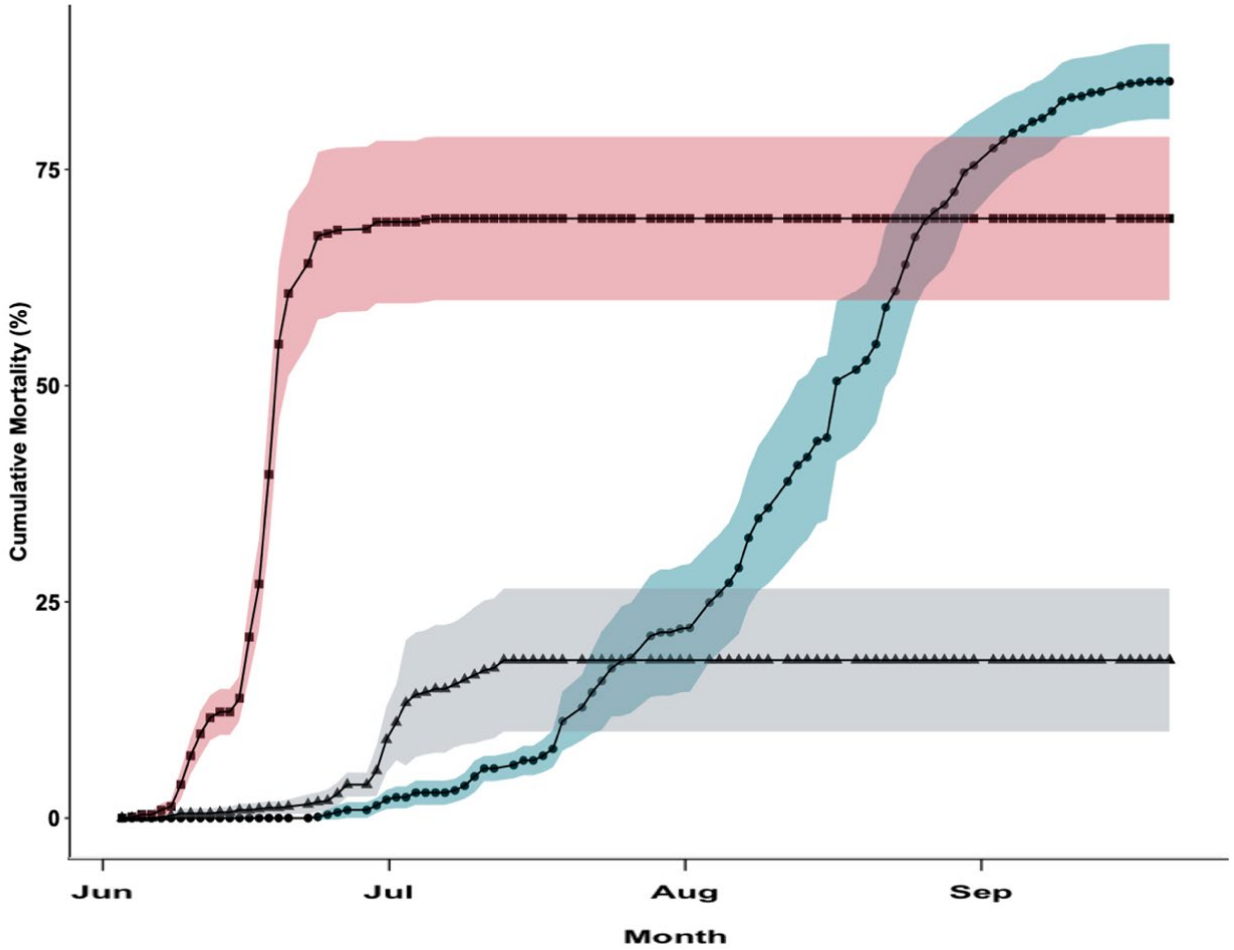


**Figure A2.** Cumulative mortality rates of larval Bluehead Suckers after hatching at three acclimation temperatures (8°C circles; 18°C triangles; 28°C squares). Shaded areas represent the standard deviation from the mean cumulative mortality.

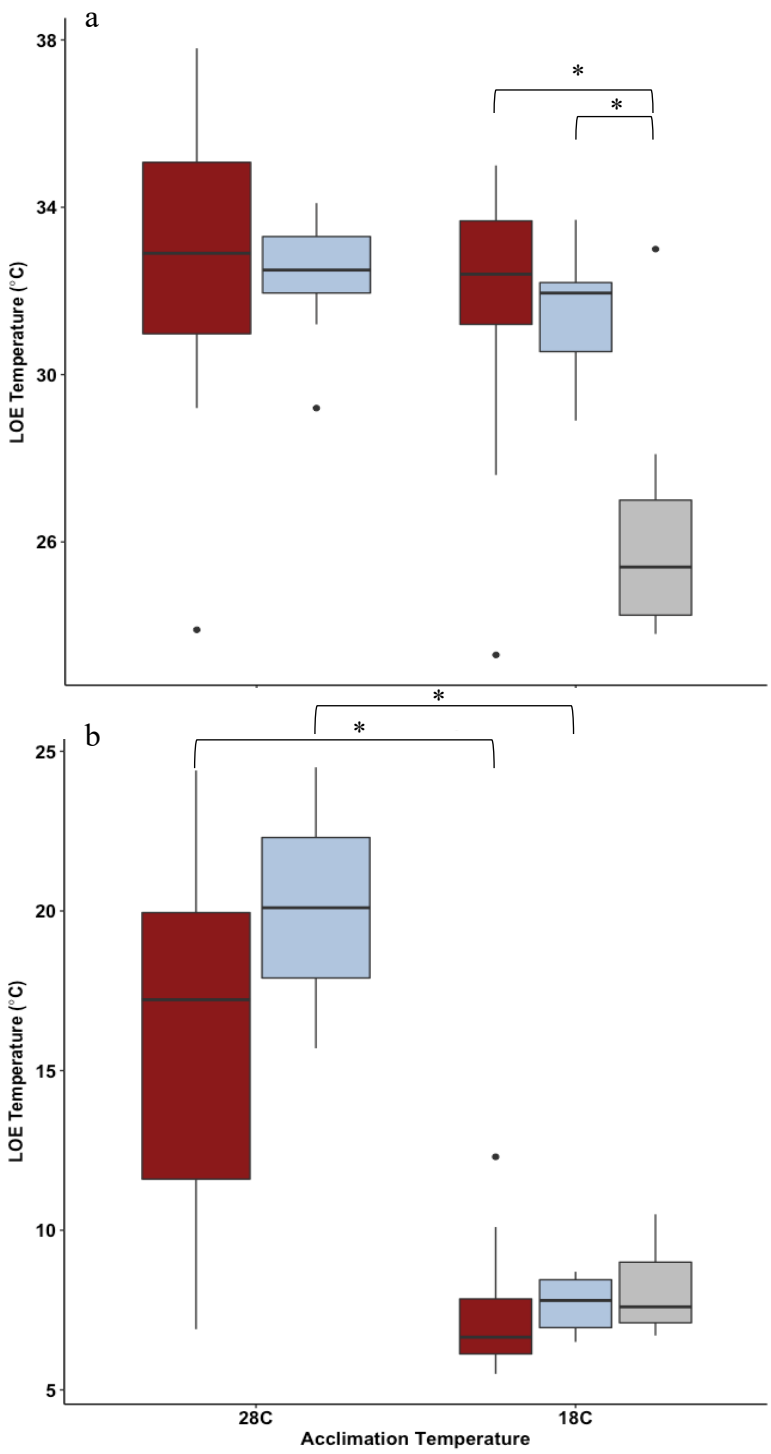


**Figure A3.** A) Critical thermal maxima and b) critical thermal minima of Bluehead Sucker at two acclimation temperatures (28°C and 18°C) and three fish densities (red = 1 fish, blue = 10 fish, gray = 25 fish). Bars with asterisks represent significant differences between the densities or temperatures ($p < 0.05$).

**Measurements of Thermal Tolerance and Cortisol From Cutthroat Trout After Magnesium Chloride Exposure**

*T.B. Riepe*

## Introduction

Magnesium chloride-based liquid deicers are used throughout Colorado to reduce the amount of salt and sand mixtures during the winter months. Current literature reviews suggest that using $MgCl_2$ based deicers are unlikely to produce any adverse effects to aquatic species because the compound is diluted through runoff before reaching the stream. The Colorado Department of Transportation (DOT) indicated in an experiment with boreal toad tadpoles, typically found in areas where deicer is used, that over a 96 hour toxicity test with 0.1% concentrations mortality was zero (Lewis 1999, 2001). In the same experiment, the concentration of deicer required to cause 50% mortality over 96 hours was 32% and with Rainbow Trout was 1.4%.

Mortality toxicity tests provide critical information to understand the worst-case scenario under which the target species would not live after exposure. The lethal concentration is used to determine the maximum lowest standards allowed. The DOT reports that the concentration of $MgCl_2$ in a stream after application is typically low and will not cause direct mortality. However, there have not been any studies determining the amount of sublethal stress the exposure of $MgCl_2$ causes to fish. The capacity of fish to cope with stressors is a main component of the normal functions of fish, because stressors affect the reallocation of energy towards different defense and behavioral mechanisms (Alfonso et al. 2020). The functional definition of stress has been noted as “a condition induced by a stressor that evokes an endocrine response that could be beneficial as well as disadvantageous” (Gorissen and Filk 2016). Responses to stress are not inherently negative, but the associated physiological and behavioral adjustments that fish need to go through can cause negative influences, especially when the stress is repeated, or other stressors are present (Barton 2002). Thus, it is important to first understand how $MgCl_2$ exposures generate a stress response to fish and how added stressors also affect fish. Here we sought to determine the level of stress fish are under after $MgCl_2$ exposures and the effect of

multiple stressors by measuring free water-borne and blood serum cortisol, the stress steroidal hormone.

**Methods**

One-year old Cutthroat Trout (*Oncorhynchus clarkii*) were obtained from the Glenwood Springs Hatchery (Glenwood, CO, USA) and transported to the Aquatic Toxicology Laboratory (Fort Collins, CO, USA). Fish were maintained in temperature-controlled troughs at 13°C until used for the experiment. Tanks were cleaned every two days and fish were fed Bio-Oregon #2 size feed at a 3% maintenance diet every day.

Chemical stock solutions of $MgCl_2$ exposures were prepared by dissolving calculated amounts of analytical reagent grade toxicant in deionized water. Chemical stock solutions were delivered to the diluter via peristaltic pump with food-grade vinyl tubing at 2.0 mL/min. $MgCl_2$ exposures utilized a serial dilution of concentrations with the diluter delivering five concentrations with a 50% dilution ratio and an exposure control that included the source water. Each exposure concentration had four duplicates of each concentration represented and was delivered at a rate of 40 mL/min. Nominal $MgCl_2$ concentrations delivered to Cutthroat Trout were 0, 53.75, 107.5, 215, 430, and 860 mg/mL. Diluters were monitored daily to ensure proper operation.

The exposures studies were completed twice with two fish densities (n = 1 fish, n = 10 fish). At the start of each test, Cutthroat Trout were distributed one at a time with a hand net for each exposure concentration until each tank contained the desired number of fish. Tanks consisted of 5 L tanks located in a water bath supplied with 13°C water to maintain temperatures inside of the tanks at 13°C. During the exposure, 50 mL water samples were collected four times a day, every six hours, to determine actual magnesium and chloride concentrations delivered to each tank. We also collected 15 mL of water for cortisol analysis at every sampling event and kept frozen till use at -20°C. Water samples were also collected prior to the experiment starting in each tank and at the source water at every sampling event. At the end of each experiment fish, were euthanized, weighed (g), and measured (mm). We also collected blood plasma after the

high-density trial through the caudal vein, centrifuged the sample in a vacutainer containing a clot activator and gel for serum separation, and froze the serum sample at -80°C until use.

We followed Friebertshauser et al. (2020) for cortisol extraction from water samples. Water samples for free cortisol were filtered through EtOH primed C-18 cartridges (Sep-pak, Waters Technology Corporation, Milford, MA). Cortisol was eluted from the cartridge with two 2-mL washes of 99.5% ethyl acetate and evaporated under nitrogen gas. Residues were eluted with EIA buffer following the ELISA kit for extractions of cortisol (Cayman Chemical, Ann Arbor, MI). We also followed this kit to determine concentration of cortisol from blood plasma samples.

Actual chloride concentrations were confirmed using a flow-injection analysis (FIA) on a Latchet instrument. Stock chloride solutions were prepared in the range of 0 - 100 mg/mL and were included in each run of the FIA. We used a mercury-based color reagent dissolved in methanol thiocyanate and nitric acid and distilled water for the carrier which were provided to the instrument by use of a peristaltic pump. Samples from an automated sampler were injected into an 18.5-µL sampling loop, and subsequently into the water carrier stream by a two-position sampling valve. Injected samples were combined in a mixing coil and the color reagent solution was added then mixed with the sample-water carrier stream. Samples were measured in the spectrophotometer at 480 nm. Duplicates, spiked samples, and internal and external verification were included in each run to ensure accuracy of the instrument and our results. Magnesium analysis has not yet been analyzed but will be using an inductively coupled plasma spectrophotometer.

**Results**

There were no mortality events during this experiment among the Cutthroat Trout, as expected. Analysis of all the samples is ongoing and we are still analyzing the concentration of cortisol samples in water and blood samples. We are also still analyzing the results from the critical thermal maxima trials and do not have any results to report at this time.

All samples prior to the experiment and from the source water (dechlorinated municipal tap water) indicated that the water included a low amount of chloride, therefore we adjusted all water samples to reflect this result. Results from the chloride analysis in the FIA indicate that our actual concentrations were similar to the nominal concentrations as expected for the low-density trial (n = 1), but we did observe a few tanks in which the concentration was higher than expected for the high-density trial (n = 1; Figure B1).

**Discussion**

This study is ongoing, but we expect to observe increased cortisol levels as a function of increased $MgCl_2$ concentrations and density. We also expect to observe lower critical thermal tolerance as concentrations of $MgCl_2$ increase. The results from this study will directly inform biologists working with Cutthroat Trout in areas where high volumes of $MgCl_2$-based road deicers are used and aid in determine mitigation efforts or future regulatory standards.

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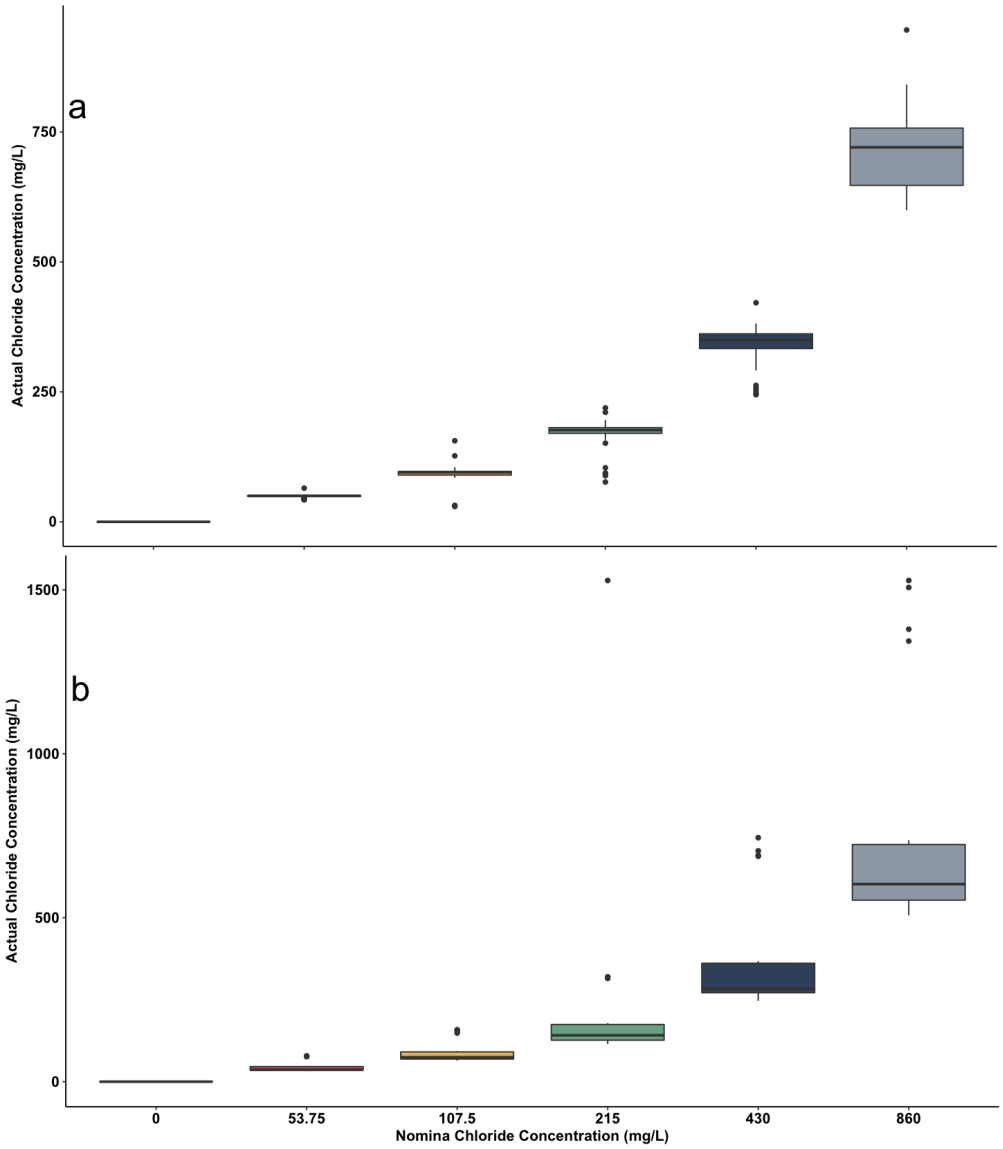


**Figure B1**. Actual chloride concentrations (mg/L) among the six exposures for low-density (n = 1 fish per tank, a) and high-density (n = 10 fish per tank, b).

**Effects of Zinc on the Population Growth of the Midge (*Paratanytarsus* sp.)**

*P. Cadmus*

## Introduction:

The family Chironomidae (non-biting midges) is the most diverse and widespread amongst aquatic invertebrates. Over 20,000 different taxa are known with a worldwide ubiquitous distribution, including Antarctica. Chironomids fill ecologically significant and diverse roles and are an important prey species to most fish and predatory invertebrates. The larvae provide a vital function as primary consumers feeding on algae, detritus, and other smaller invertebrates. Ecologists have used chironomids to characterize overall quality of lentic and lotic systems where the density and diversity of some species can serve as an indicator of toxicants within aquatic ecosystems (Merrit et al. 2008). Despite an amazing diversity of feeding guilds and functional niches within this family, the members of Chironomidae are often lumped into a single family or tribe level resolution in biomonitoring due to the difficulty of identification. This difficulty might also inadvertently lead to reluctance to use field collected chironomids in aquatic toxicology experiments leaving only a few laboratory-cultured species included in toxicology research.

Several large bodied, often tolerant, chironomid taxa have been used widely as test organisms (e.g. *Chironomus riparius*, *Chironomus tentans*). These are available from numerous mail order suppliers, have a very robust survival rate in the laboratory setting, and are often the sole taxon representing chironomids in the species sensitivity distributions used to derive water quality standards. According to Section 304(a)(l) of the Clean Water Act of 1977 (P.L. 95-217) the Environmental Protection Agency (EPA) is required to publish water quality criteria "accurately reflecting the latest scientific knowledge on the kind and extent of all identifiable effects." Despite this mandate to use the best available science data informing most standards is limited and lacking ecological realism (Duggan and Kotalik 2020). Most criteria are informed only by standardized single species toxicity trials that examine only aqueous exposures and use only laboratory cultured or mail order organisms in the most tolerant life stage. Historically these tests have encompassed limited diversity of species. Guidelines of "acceptable" data for inclusion in standard derivation require an unnaturally limited amount of mortality in controls and minimal stochasticity between replicates. This well-intended requirement of strict control and reproducibility unintentionally excluded most studies with strong ecological relevance. For

this reason, aquatic insect toxicity trials that use larger, more tolerant, life stages are more acceptable (Cadmus et. al 2020). Development of new ecologically sound methods is expensive and risky and avoided by most for-profit firms and most government agencies. Use of sensitive species or field collected organisms is often expensive or risky for firms or agencies conducting laboratory trials. Colorado Parks and Wildlife has maintained a culture of the genus *Tanytarsus (*later named *Paratanytarsus)* since 2007 in hopes that this small bodied chironomid could represent other sensitive chironomids in species sensitivity distributions.

Zinc is a common heavy metal that naturally occurs in the environment and is essential to all living organisms. However, at high concentrations, zinc is toxic. The occurrence or presence of zinc within freshwater systems is largely influenced by anthropogenic disturbance, primarily mining. Colorado has over 23,000 abandoned mines including 1,800 miles of impaired streams which are contributing to a total median value loading rate of 905 µg/L statewide (Graves et al. 2017). A marked disparity exists between occupancy of aquatic insects in nature and what would be predicted by water quality standards. Under-protective zinc criteria may be a possible explanation.

The 1980 EPA Ambient Water Quality Criteria for Zinc (USEPA 1980) highlighted a study using the chironomid *Tanytarsus dissimilis* that demonstrated an LC50 of 36.8 µg/L following a 10 day exposure (Anderson et al. 1980). This national criteria document prescribed a Final Chronic Value of 47 µg/L at a hardness of 50 mg/L (USEPA 1980), failing to protect the *Tanytarsus* genus. Forty years later Colorado State's Zinc Chronic Value has crept up to 80 µg/L at a hardness of 50 mg/L $CaCO_3$ despite *Paratanytarsus* sp. or members of Tanytarsini (tribe) being documented in reference (low levels of pollution) streams across the state. Numerous site-specific water quality standards also excluded consideration of *Paratanytarsus* sp., by removing the Anderson et al. (1980) research from Species Sensitivity Distributions due to insufficient duration of exposure (10 d). This despite the widely accepted biological assumption that organisms dead at 10 days of exposure would remain dead at 30 days of exposure.

The purpose of this study was to better characterize the zinc toxicity threshold for chironomids using a laboratory cultured organism more pertinent to Colorado's aquatic

ecosystems. *Paratanytarsus* sp. has been recognized across numerous organizations and across numerous continents as a potentially ideal toxicology test organism with cosmopolitan distribution (Encina et al. 2020; Gagliard et al. 2015). Slight modification of traditional aquatic toxicology methods have been developed to add ecological relevance in a way that traditional laboratories could successfully reproduce. Vessel size of each experimental unit was markedly increased to prevent density dependent effects observed by growing *Paratanytarsus* sp. in smaller aquaria. The slow lotic flow of hyporheic zones or stream margins was approximated using only aeration stones and an angled septum. Large aerial habitat volume was provided as terrestrial habitat for adults. A consistent number of eggs was added to each experimental unit each day for 30 days at the start of the experiment. This was done to increase the likelihood of mate pairing when populations are low and when emergence is not synchronous. This multiple day start also reduced an accidental "family" or inbreeding effect of starting with limited number of organisms from a limited number of parents on a single day. Exposures were conducted using flow-through diluter systems to avoid shortfalls associated with static renewal. Exposure durations were multigenerational, extended to 137 days (2019) and 84 days (2020). This ensured inclusion of all life stages (egg, numerous instars, pupa, adults) and possibly stressful behaviors essential for life (*e.g.* mating, oviposition, emergence). Inclusion of multiple generations unfortunately adds stochasticity between replicates. However, it allows the experiment to examine population growth not simply survival.

## Methods and Materials

### *Culture*

In 2007 a *Paratanytarsus* sp. culture was started using organisms collected from Clear Creek (Idaho Springs, CO, USA), a tributary to the South Platte River. Larvae organisms were reared in 18 L aquaria with ceramic tiles and sand for sediment substrate. Each aquarium received 40 mL/minute flow-through dechlorinated tap water. Pipe stands held depth at approximately 66% of the tank height. The excess flow-through water drained out of each aquarium through a pipe stand with a mesh filter attached to the top to prevent emigration. The aquaria were covered with lids or nets. To harvest adults, clear acrylic lids with 59 mm holes and funnels (60 mm diameter) were fixed above the hole. This acted as a funnel trap commonly employed by insect sampling nets. Over the upside-down funnels were smaller aquaria flipped

upside-down to capture adults. The positive phototaxic nature of the test organism drove emerging chironomids upward towards wide spectrum grow lights hung above the tanks. Emerging adults flew upwards through the funnel traps and were contained in each overturned aquaria. To harvest eggs, petri dish filled with water were placed in each trap to allow adults to oviposit in water (Figure C1). Egg masses were laid in long strings of eggs (Figure C2). Eggs can be counted under a low magnification dissection microscope. Cultures and each experimental unit of this study were fed 1 cc artificial rotifers (AZOO 9 in 1 Artificial Rotifera. AZOO MFG., Taiwan*)* in addition to natural algal growth within each tank. When aquaria became overpopulated or when natural biofilm transitioned from being diatom dominated to green algae dominated an aquarium was retired from use. All adults or eggs produced thereafter were moved to a new aquarium.

*Zinc Exposure*

Aquatic – terrestrial habitat was simulated using 492 L high density polyethylene rectangular tanks (61 x 76 x 107 cm. R243042A. Chem-tainer Industries. West Babylon, NY, USA). Pipe stands held depth at 42.5 cm (~183 L). The excess flow-through water drained out of each tank through the pipe stand. A mesh filter attached to the top of each pipe stand prevented emigration and immigration. Each exposure tank contained a clear plexiglass septum set at the bottom of the tank at a slight angle with an air stone at the low end to simulate a lotic system (Figure C3). A mesh top emergence trap (Cadmus et al. 2016; Constructed with NZ11 White Mesh. Apex Mills. Graham, NC, USA) was used as a lid which prevented immigration and emigration from occurring and provided habitat for adults (Figure C4). Supplemental feed of a 9-1 artificial rotifer was added to each tank biweekly which was the primary food source in addition to the natural growth of algae within each tank. Exposure tanks received a 15:9 hr light:dark photoperiod from wide spectrum lights. To ensure equal photosynthetic potential for algal growth a light meter was used to position lights to achieve equal lighting within each test tank. Total and dissolved zinc, D.O.%, D.O. mg/L, pH, specific conductivity, alkalinity, and hardness and temperature were assessed across all 24 experimental units weekly.

Zinc ($ZnSO_4$ x $7H_2O$) stock solution was delivered by peristaltic pump to a gravity fed serial diluter which delivered 40 mL/minute municipal dechlorinated tap water and a gradient of toxicant to each experimental unit. In 2019 (137 days; 9 Sept 2019 – 16 Jan 2020) exposures of

180µg/L, 90µg/L, 45µg/L, 22µg/L, 11µg/L, and 0µg/L were assigned to 24 exposure tanks (4 reps per treatment level) and were observed at 241.5µg/L, 120.6µg/L, 57.7µg/L, 29.1µg/L, 12.2µg/L, and values below our detection limit (BDL), respectively. These values were designed to straddle the national and state chronic values and approach the site-specific Zn criteria for the Arkansas River Headwaters near Leadville (CO, USA. >180 µg/L). In 2020 (84 days; 28 Dec 2020 to 4 March 2021) exposures of 20 µg/L, 10 µg/L, 5 µg/L, 2.54 µg/L, 1.25 µg/L and 0 µg/L and were observed at 22.77 µg/L, 11.37 µg/L, 7.31 µg/L, 2.86 µg/L, BDL and BDL respectively. After the diluter concentrations were confirmed by ICP-MS the exposures were initiated by delivering eggs to each experimental unit every day for the first 30 days of each experiment. Eggs oviposited in Petri dishes (Figure C1) in the *Paratanytarsus* sp. culture were tallied, divided equally under a dissecting microscope into 24 beakers and then randomly assigned to each exposure tank daily. A total of 413 eggs were delivered to each experimental unit in the 2019 experiment and 248 eggs were delivered to each experimental unit in the 2020 experiment. Based on preliminary studies using *Paratanytarsus* sp. since 2007, we estimated the lifecycle at this temperature to be 14-18 days. At 14.0-17.0 °C in each exposure tank across both studies we estimate the 2019 exposure included 6 to 9 generations and the 2020 exposure included 3 to 6 generations.

Preliminary experiments using similar and smaller vessels suggested that space limitation was likely with this taxon in any multigenerational experiment. Experiments were conducted for longest logistically feasible duration that ensured density of organisms does not limit survival and recruitment. In an effort to estimate the populations of each experimental unit, preliminary studies explored lethal emergence traps, non-lethal emergence traps, Hester Dendy (1962) samplers, laser counters and photo analysis. In the 2019 and 2020 experiments presented here we employed only photo analysis to estimate *Paratanytarsus* sp. populations. Experiments were terminated when the rate of population increase was no longer increasing at an increasing rate (AKA inflection point of exponential population growth). Every 14 days, photos were taken (Apple iPhone 2020se, 12 megapixels. Apple Inc. Cupertino, CA, USA) under uniform light from uniform position (centered and 61 cm from water level) by reaching under the emergence net of each exposure tank. MacBook Photos v4.0 was used to crop photos. The cell counting program Cell Profiler v 3.1.9 (Broad Institute of MIT and Harvard, Cambridge, MA, USA) was

then used to count unique objects as surrogate for population. The processes “ColorToGray” "Image Math" and "Identify Primary Objects" (See settings on Figure C5) to identify the tube structures that were 2 to 6 pixels in size. This matched the measured size of a *Paratanytarsus* sp. case/tube. The image analysis accuracy was then checked manually by comparing the number of primary objects identified (tube structures) by CellProfiler with the actual counts confirmed by a human within an image on a zoomed subsample of each photo. Thresholds and pixel minima and maxima were adjusted until the observed accuracy and the number of primary identified objects within the program were within 25% of the hand human counted population.

Five hours before ending the experiment, bottle traps were attached to emergence traps to capture adults. Pulse Amplitude Modulated Fluorimetry (BenthoTorch. bbe Moldaenke GmbH, Schwentinental, Germany) was used to assess composition of biofilm in each exposure tank (nine readings at uniform locations equally spaced on the clear acrylic septum). All liquid and contents were processed through a 250 µm mesh (triangular pore size. NZ11 White Mesh. Apex Mills. Graham, NC, USA) filter lining a 250 µm wire sieve (Figure C6). Contents were preserved in ethanol for enumeration at a later date.

**Results and Discussion:**

*Water Chemistry*

Zinc concentrations were consistent and well matched to our targets (Tables C1 and C2). Physical chemistry observations were consistent with what is observed in Colorado headwater streams.

*LC20 and experimental duration*

Enumeration of preserved organisms showed a clear dose response (Table C3 and C4). After exposure for 137 d we observed an EC20 of 11.5 µg/L zinc. Controls had *Paratanytarsus* sp. populations as high as 3936 in an experimental unit. Experimental units are still being picked from the 2020 experiment, but preliminary analysis suggests an EC20 close to the 2019 experiment. These results are in agreement with Anderson et al. (1980) given the difference in duration and the use of an EC50 rather than an EC20. Compared to the state standard of 80 µg/L at our observed hardness, some chironomids were not well protected under the Colorado State

standards for aquatic life.

Guidelines for acceptable data for inclusion in chronic criteria (e.g. ASTM or USEPA) require a minimum number of days (30 d) because it would lead to falsely high pollution limits if everyone ran chronic tests at 5 days, thus mortality would be underestimated. In the case of Anderson et al. (1980) the zinc mortality observed after only 10 d occurred well below the standards that it was excluded from. It should have been the opinion of policy makers to look past the "insufficient duration" and acknowledge such a low toxicity threshold, in a high-quality experiment, should inform or drive the standard. Here we sought the taxon used in Anderson et al. (1980) and extended the study to 137 and 84 days. Extending studies beyond the 30 day limit is encouraged because it more realistic. However, in experiments evaluating exposure of multiple generations of organisms, competition limits growth as the population nears a carrying capacity of the food or space. Figure C7 shows a hypothetical population growth curve of a control (blue) population and several exposed populations over time. In this hypothetical situation, the control population grows without the stress of a hypothetical toxicant; twice as fast as the low treatment (red); three times as fast as the medium level treatment (green); maybe 4 times faster than the high-level treatment group (yellow). It took more or many more days for our exposed populations to reach carrying capacity (dotted line). If the experiment was ended arbitrarily, or any time after 'a,' it would appear that an expensive long term multigenerational study found no effects of the hypothetical toxicant even in the highest treatment level. Without being able to measure the population of small bodied organisms like midges throughout the experiment the scientist is clueless that all treatment levels had retarded growth relative to the control population. An ideal time to measure is possibly the inflection point of the growth rate of the fastest growing experimental unit (see "i" Figure C7). Exposures of 4 day and 30 day are convenient for the work week. A duration smaller than a full life cycle for any species is of limited value and surely fails to explore implications at higher levels of biological organization (population, community, ecosystem). For this reason, our experimental duration was extended to as many generations as feasible before the population outgrew the habitat that our laboratory could host.

*Non-lethal estimation of populations*

Preliminary experiments worked to devise methods to determine population size without disturbing the experiment. Emergence nets with live or lethal bottle collectors were of limited use. Mating in this species appeared to be asynchronous for the first 2 months. An observation of adults at a scheduled frequency had potential to represent the larger populations. When extended past two months, synchronous emergence within tanks was obvious but inconsistent between tanks. Use of traps infrequently was assumed to miss the stochastic emergence events. Use lethal bottle traps every day would have become a population sink. Hester Dendy samples or another passive subsampling substrate removed occasionally had potential to estimate population growth but the research and development of establishing a model became prohibitive. In this study photo analysis was used to estimate population in this experiment solely to identify the inflection point (Figure C7) of the population growth curve of the most productive experimental unit.

*Algal composition*

Algal composition on the final day of the 2019 experiment showed effects of grazing by *Paratanytarsus* sp., showed reduced algal colonization in higher treatments and showed increased green algae competitiveness in higher treatments (Figure C8). Control (0µg/L) populations averaged 2,714 *Paratanytarsus* sp. while the 11µg/L treatment group averaged 253 organisms. This trend continued with increasing concentrations (Table C3). Grazing pressure in the controls reduced diatoms. In higher treatment levels, were grazing pressure was reduced, colonization of diatoms (nutritious algae) decreased with increasing Zn, but well above the toxic threshold estimated for *Paratanytarsus* sp. Green algae began to outcompete diatoms and cyanobacteria in the highest treatment levels. Diatoms have a higher nutritional value than green algae and cyanobacteria (responsible for harmful algal blooms). Future studies should examine if grazing pressure by algivorous fish and invertebrates or toxicants can elicit a community shift between these taxonomic groups because diatoms are nutritious.

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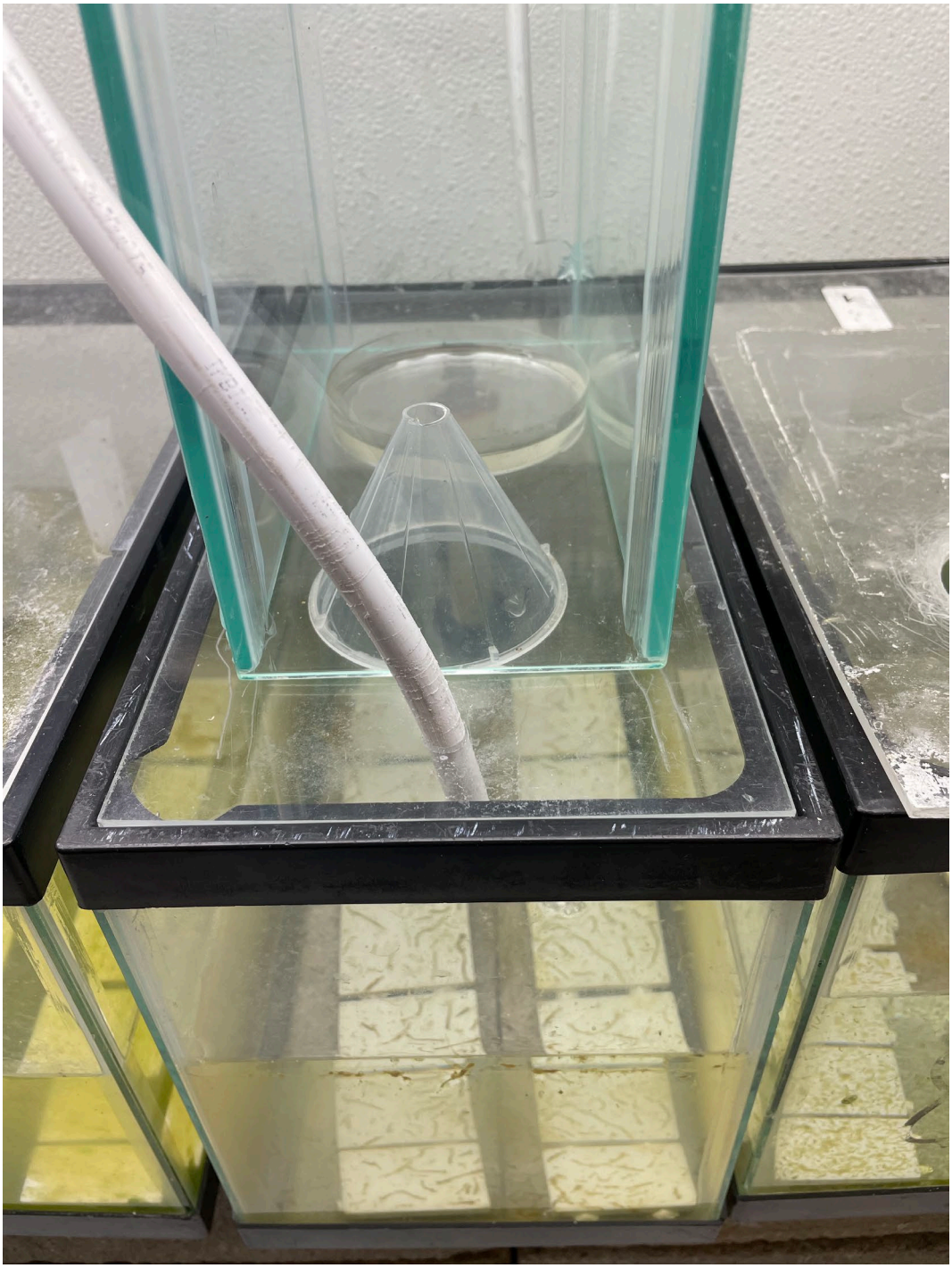


Figure C1: *Paratanytarsus* sp. Culture Aquarium. Lids with holes cut in them and funnels fixed to each hole acted as a funnel trap. Each aquaria had a smaller tank flipped upside down on top of the funnel trap to contain emerged adults. Adults laid eggs in the Petri dish containing water.

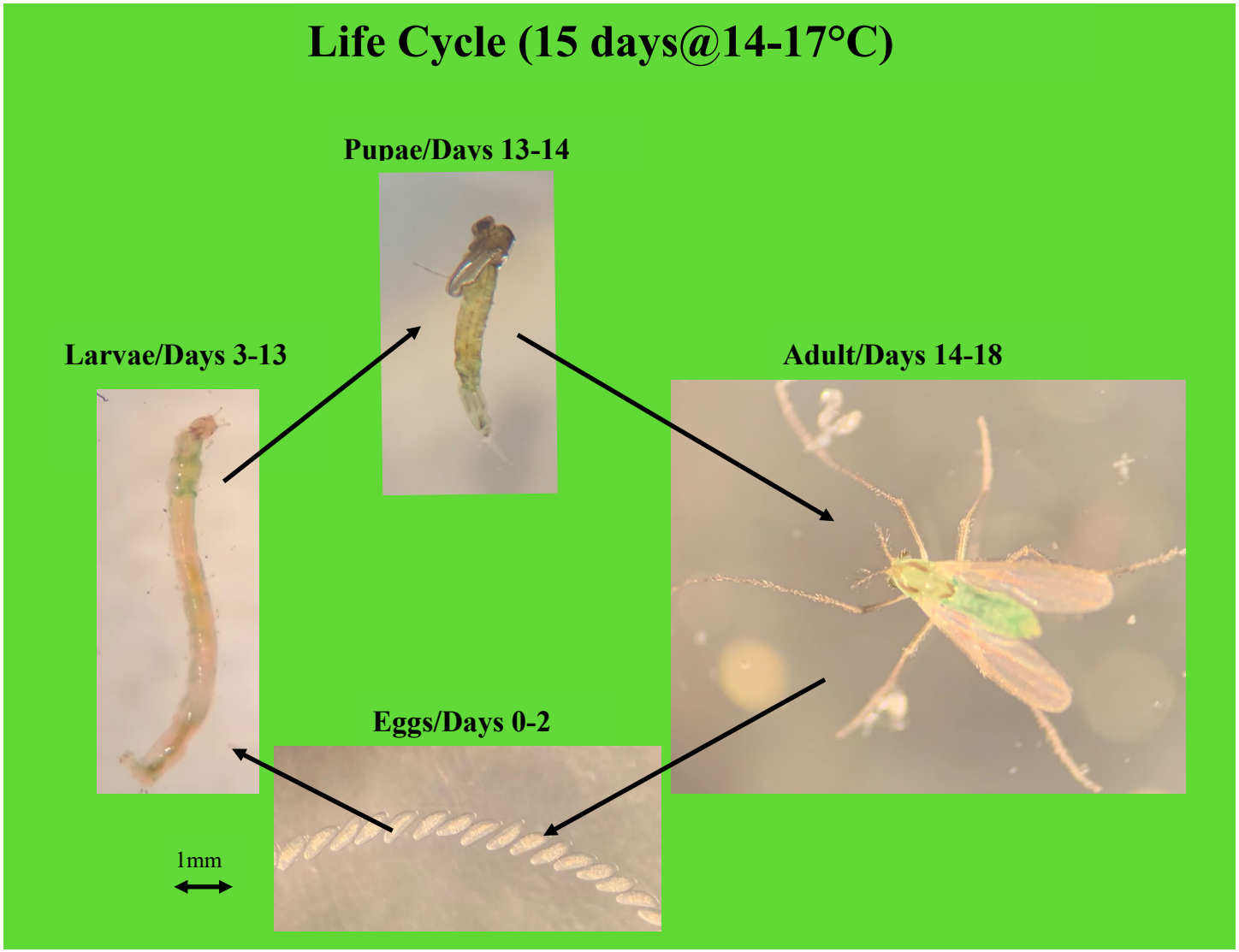



Figure C2: Life Cycle of *Paratanytarsus* sp.. *Life stage durations* when held at 14-17 Celsius at a hardness of 50 mg/L. *These durations are based on CPW's observations since 2007*

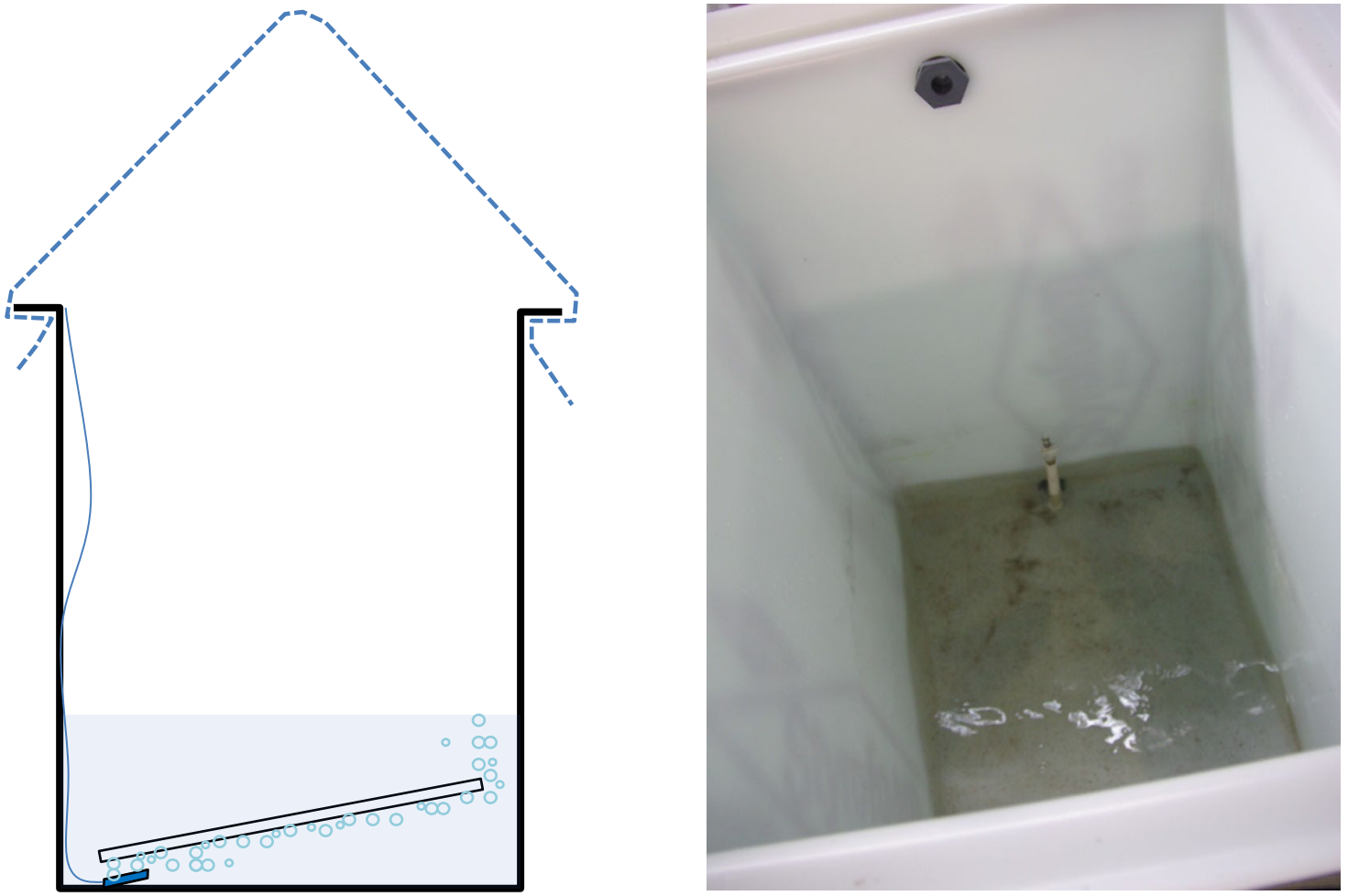
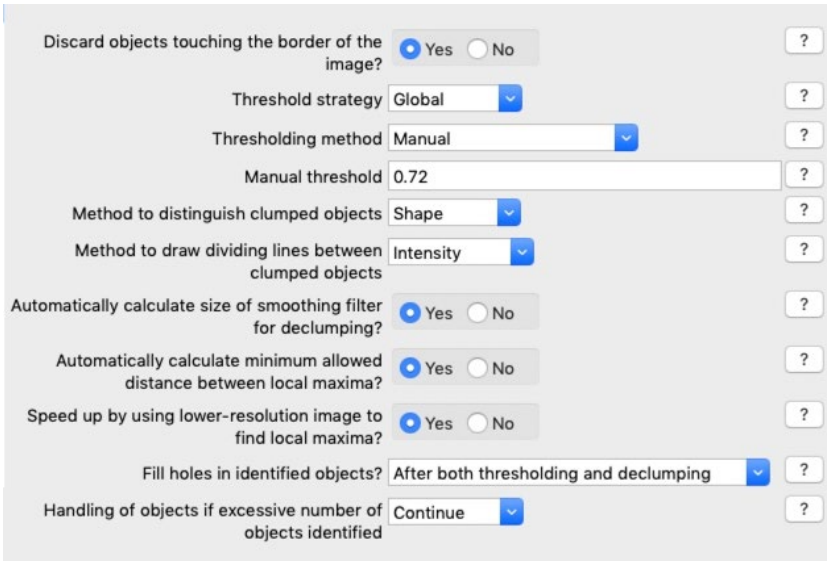


Figure C3: Aquatic-terrestrial exposure tanks. (Left) Exposure tank diagram showing the air stone and clear plastic septum along with the emergence net top. (Right) Photo of clear acrylic angled septum and water level during the experiment.

Figure C4: Aquatic-terrestrial exposure tanks. Exposure tanks were assigned such that each of the four blocks (foreground to back ground) had one replicate of each treatment level randomly assigned (right to left). Gravity fed serial diluter shown in the center of photo.

a.


b.




c.

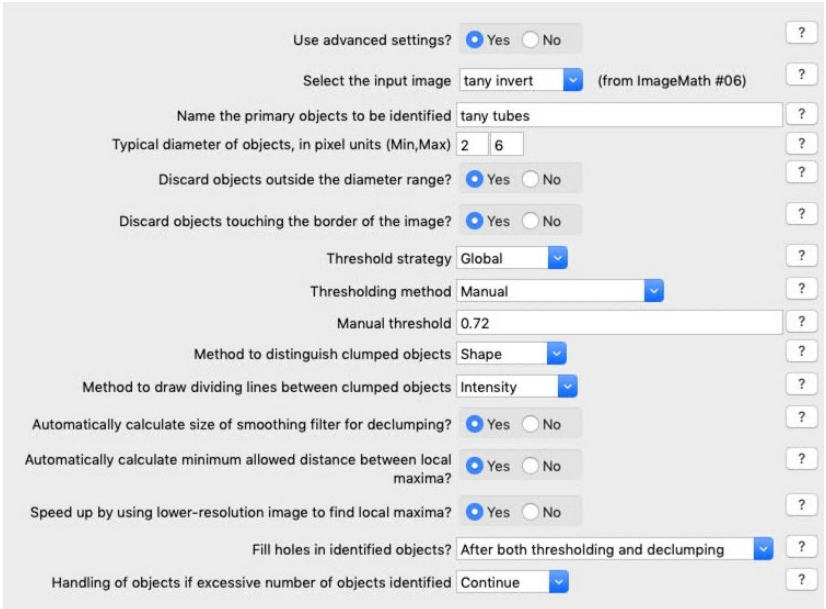



Figure C5: Setting used within CellProfiler v3.1.9 - Setting for "ColoradoToGray" (a) "Image Math" (b) and "Identify Primary objects" (c) functions within CellProfiler.

Figure C6. Processing of samples on final day. All organisms and detritus retained in a 250 µm sieve was preserved in 80% ethanol.

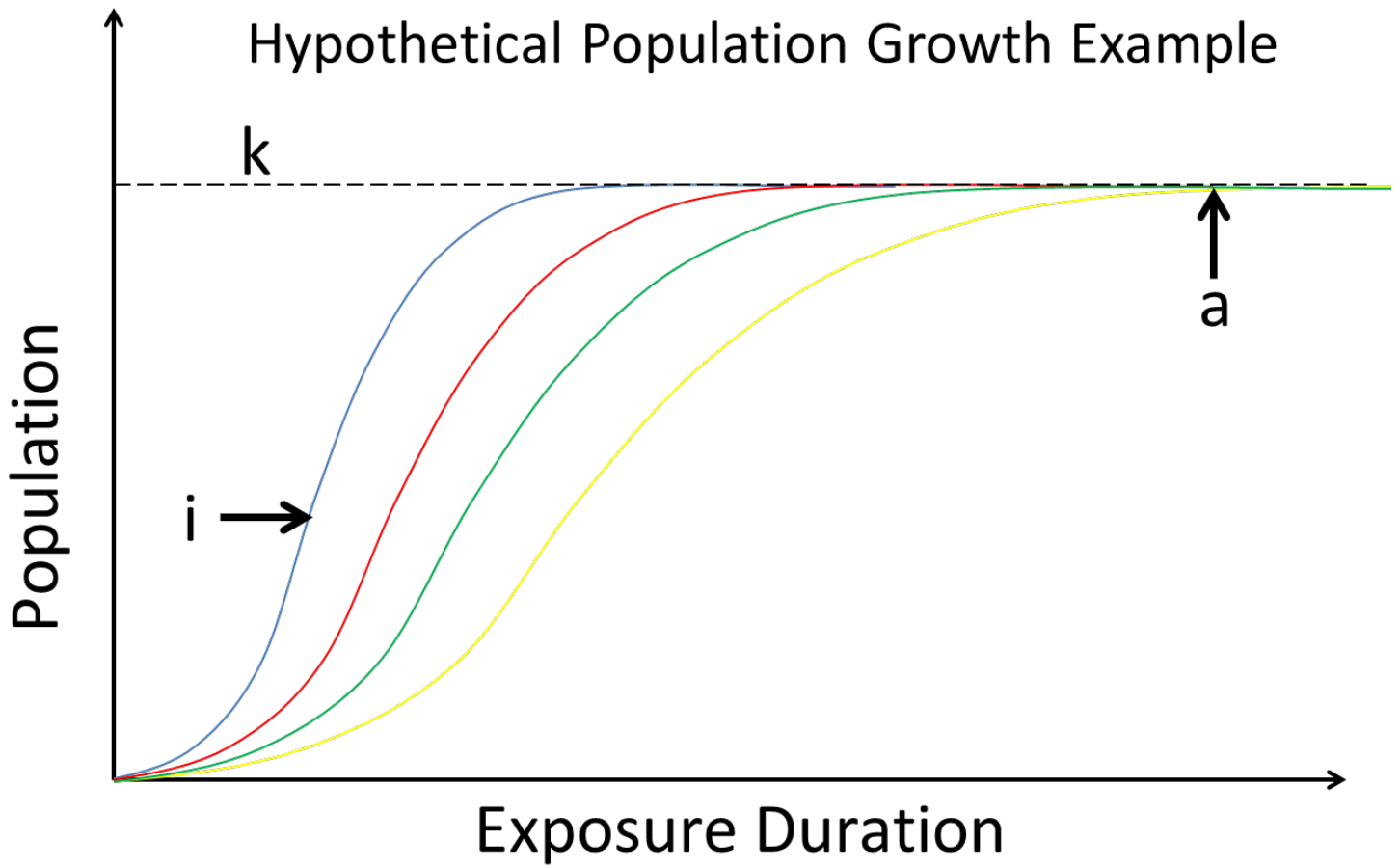



Figure C7: Hypothetical Population Growth Curves. These experiments were ended not on a predetermined date but instead were terminated when an inflection point (i) was observed in the fastest growing replicate. This avoided density dependent effects as populations neared carrying capacity (k). Running an experiment too long (a) or too short (< full life cycle) risks underestimating a toxic threshold.

z

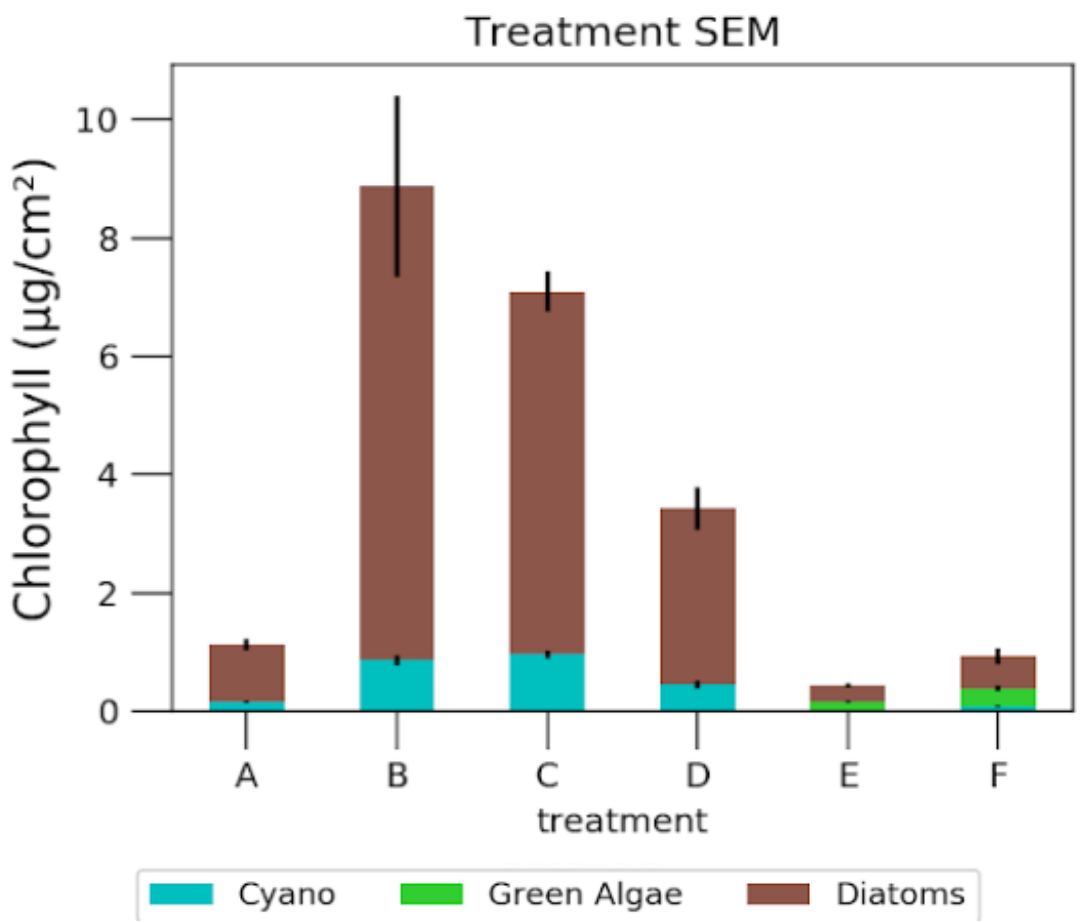



Figure C8: Algal composition on final day of the 2019 experiment. Chlorophyll florescence from three major algal taxa Cyanobacteria (Cyano), Green Algae and Diatoms observed within each treatment level A=Control 0µg/L B=11µg/L C=22µg/L D=45µg/ E=90µg/L F=180µg/LZn. Control (A) populations of *Paratanytarsus* sp. averaged 2,714, while 11µg/L treatment group averaged 253. Algae in the control (A) was reduced by intense grazing by *Paratanytarsus* sp. Reduced survival of Diatoms (nutritious algae) decreased with increasing Zn. Green algae began to outcompete diatoms and cyanobacteria in the highest treatment levels.

Table C1: Water Quality 2019. *= Below Detection Limit

| Treatment | Nominal Concentration Zinc µg/L | Observed Concentration Average Dissolved Zinc µg/L ± SD | Average Temp ± SD (Celsius) | Average D.O. %±SD | Average D.O. mg/L ±SD | Average pH ±SD | Average Specific Conductivity (us/cm) ±SD | Average Alkalinity (mg/L) ±SD | Average Hardness (mg/L) ±SD |
|---|---|---|---|---|---|---|---|---|---|
| Control | * 0.00 | * -0.43 ±0.28 | 15.76 ±2.24 | 105.24 ±0.69 | 8.80 ±0.52 | 7.69 ±0.26 | 167.62 ±0.37 | 35.18 ±2.14 | 54.17 ±3.21 |
| Low | 11.5 | 12.16 ±2.18 | 15.79 ±2.07 | 104.96 ±0.76 | 8.76 ±0.51 | 7.84 ±0.18 | 167.50 ±0.32 | 35.80 ±1.18 | 52.58 ±3.69 |
| Mid-Low | 22.87 | 28.76 ±4.12 | 15.75 ±2.09 | 105.25 ±0.92 | 8.81 ±0.54 | 7.87 ±0.08 | 167.31 ±0.37 | 35.42 ±1.83 | 54.07 ±3.08 |
| Mid | 45.49 | 56.10 ±8.19 | 15.70 ±2.09 | 105.19 ±0.69 | 8.80 ±0.50 | 7.88 ±0.08 | 167.81 ±0.46 | 35.05 ±2.44 | 52.91 ±3.55 |
| Mid-High | 90.49 | 117.98 ±11.60 | 15.81 ±2.07 | 105.61 ±1.50 | 8.80 ±0.56 | 7.78 ±0.19 | 166.25 ±2.95 | 35.07 ±2.25 | 54.53 ±5.97 |
| High | 180 | 243.07 ±16.37 | 15.90 ±2.12 | 106.03 ±7.73 | 8.81 ±0.57 | 7.88 ±0.13 | 166.65 ±2.70 | 35.18 ±2.24 | 55.71 ±5.89 |

Table C2: Water Quality 2020. *= Below Detection Limit

| Treatment | Nominal Concentration DIs Zinc µg/L | Observed Concentration Average DIs Zinc µg/L ± SD | Average Temp (Celsius)± SD | Average D.O. %±SD | Average D.O. mg/L ±SD | Average pH ±SD | Average Specific Conductivity (us/cm) ±SD | Average Alkalinity (mg/L) ±SD | Average Hardness (mg/L) ±SD |
|---|---|---|---|---|---|---|---|---|---|
| Control | * 0.00 | *1.29 ±2.36 | 15.87 ±0.38 | 97.83 ±1.94 | 8.16 ±0.21 | 7.51 ±0.20 | 176.29 ±8.06 | 35.50 ±1.09 | 53.95 ±1.53 |
| Low | * 1.25 | *2.71 ±2.81 | 15.83 ±0.37 | 97.57 ±2.29 | 8.13 ±0.28 | 7.60 ±0.16 | 175.93 ±7.27 | 35.45 ±1.19 | 53.67 ±1.77 |
| Mid-Low | 2.54 | 2.86 ±4.22 | 15.78 ±0.43 | 97.33 ±2.46 | 8.16 ±0.25 | 7.61 ±0.17 | 174.17 ±5.11 | 35.30 ±0.96 | 53.20 ±1.52 |
| Mid | 5 | 7.31 ±1.73 | 15.83 ±0.41 | 97.47 ±2.32 | 8.15 ±2.545 | 7.63 ±0.09 | 176.17 ±6.47 | 35.50 ±1.18 | 53.52 ±1.912 |
| Mid-High | 10 | 11.37 ±1.99 | 15.82 ±0.38 | 97.47 ±2.50 | 8.15 ±0.27 | 7.64 ±0.09 | 174.42 ±5.76 | 35.40 ±1.19 | 52.57 ±1.35 |
| High | 20 | 22.77 ±2.36 | 15.78 ±0.40 | 97.45 ±2.50 | 8.15 ±0.27 | 7.61 ±0.07 | 176.00 ±6.99 | 35.43 ±0.95 | 54.37 ±2.84 |

Table C3: Population of *Paratanytarsus* sp. 2019

| Nominal Concentration DIs Zinc µg/L | Observed Concentration Average DIs Zinc µg/L ± SD | Average Total Population 2019/2020 |
|---|---|---|
| *** 0.00** | * -0.43 ±0.28 | 2714.25 |
| **11.5** | 12.16 ±2.18 | 253.75 |
| **22.87** | 28.76 ±4.12 | 22.5 |
| **45.49** | 56.10 ±8.19 | 12.75 |
| **90.49** | 117.98 ±11.60 | 3.5 |
| **180** | 243.07 ±16.37 | 0 |

Table C4: Preliminary population estimates of *Paratanytarsus* sp. 2020. Manual enumeration of samples is pending for some replicates.

| Nominal Concentration DIs Zinc µg/L | Observed Concentration Average DIs Zinc µg/L ± SD | Average Total Population 2020/2021 |
|---|---|---|
| *** 0.00** | *1.29 ±2.36 | (2/4 Pending) 5815.75 |
| *** 1.25** | *2.71 ±2.81 | (4/4 Pending) |
| **2.54** | 2.86 ±4.22 | (2/4 Pending) 2353 |
| **5** | 7.31 ±1.73 | 3553.75 |
| **10** | 11.37 ±1.99 | 2775.25 |
| **20** | 22.77 ±2.36 | 76 |

**Reduced Predator Evasion Following a Sub-Acute (24 Hour) Sub-Lethal Exposure of Diesel Fuel to Plains Minnow (*Hybognathus placitus*), Flathead Chub (*Semotilus atromaculatus*) and Fathead Minnow (*Pimephales promelas*)**

*P. Cadmus*

## ABSTRACT

Standardized methodologies for deriving species or mean acute threshold values has historically only considered mortality of organisms in acute toxicity experiments (Acute: first four days of exposure or first two days for some small organisms). The argument against consideration of sub-lethal endpoints is the organism in nature is sure to recover after the toxic exposure. It is assumed that changes in behavior, loss of olfactory function, reproduction, loss of fitness, up-regulation of genes, and any other sublethal endpoint has full likelihood to recover after the pulse stress event is ameliorated. It is irresponsible to claim that all sub-lethal endpoints are temporary and therefore do not have population effects in acute spill events. To showcase this, we conducted a study to evaluate a behavior endpoint that is sub-lethal but immediately leads to mortality. As part of a series of experiments characterizing petroleum hydrocarbons, we exposed Plains Minnow (*Hybognathus placitus*) to sublethal levels of diesel after only 24 hours. Exposures were limited to 75 mg/L nominal diesel fuel witch was measured at only 2.0 mg/L total petroleum hydrocarbons (C10-C28) for 24 hours. In non-contaminated conditions, we then conducted predator avoidance trials in which a Plains Minnow from a control group (0 mg/L diesel fuel) and exposure group competed to avoid predation by a Green Sunfish (*Lepomis cyanellus*). Diesel exposure at 2.0 mg/L for twenty-four hours significantly reduced predator avoidance of the prey species. We repeated this design using the prey species Fathead Minnow (*Pimephales promelas*) in the presence of Yellow Perch (*Perca flavescens)*, Green Sunfish and Creek Chub (*Semotilus atromaculatus*). To explore the defensive behavior of shoaling (schooling), we reproduced the experiment using schools of four Fathead Minnows that had been exposed to diesel and four Fathead Minnows that had not been exposed to diesel in the presence of multiple predators.

## INTRODUCTION

Consumption and transportation of diesel fuel is growing in Colorado and with this growth in use is a growing risk of hazardous material spills (Alternative Fuels Data Center 2021). Colorado highways are often co-located next to rivers so diesel or gasoline is the most frequent hazardous material in spills and accident reports associated with traffic collisions. According to the Center for Western Priorities, a nonpartisan conservation and advocacy organization, 20% of >340 oil and gas (petroleum) spills that occurred in Colorado in the past year (2020) were within 500 feet of surface water (McIntosh 2021). Historically, Colorado's response and characterization of resource damage after petroleum spills was limited to counting dead fish on stream banks. A more extensive understanding of the potential effects on freshwater fish within Colorado waters can help better predict and evaluate the ecosystem effects of a diesel fuel spill in nature. Gaining a better understanding of the lethal and sublethal effects amongst freshwater fish after a diesel spill event can serve to help better inform managers of appropriate information gathering and mitigation efforts in a spill event, beyond immediate mortality counts downstream of a traffic accident.

Previous Colorado Parks and Wildlife laboratory studies and observations following spill events looked at the toxic thresholds of various freshwater fish species, periphyton, and aquatic invertebrates following acute exposures to diesel fuels. Drift or chemical avoidance was found to happen immediately when aquatic invertebrates were exposed to diesel. Similar behavioral alterations are likely in fish. Behavioral responses have potential to extirpate species from spill sites especially when fish passage precludes return when toxicant levels subside. Such behavioral endpoints are rarely characterized or considered in the development of standards, criteria or policy to protect Colorado's fish from petroleum spills. Behavior changes at sub-lethal levels can manifest into mortality including a lowered ability to avoid predation.

Parks and Wildlife studies in 2014 and 2015 investigated survival of trout to acute diesel exposure comparable laboratory conditions. Six-month old (18.1 g +/- 0.44; 122.8 mm +/- 1.1) Mt. Shasta strain Rainbow Trout (*Onchorinchus mykiss),* and Greenback Cutthroat Trout (*Oncorhynchus clarkii stomias*) fry exhibited full survival at or below pulse exposures 75 mg/L

or 100 mg/L respectively. These exposures were a single dose spike followed by four days of static exposure before replacing water. Based on chromatography assessments, most of the diesel was lost from the water column in the first couple hours of exposure. These exposure values were nominal (what was delivered) and do not perfectly reflect the true concentrations of the many chemicals in the water column. In both field and laboratory studies, petroleum hydrocarbons are quickly lost from the water column. This makes study of diesel and petroleum spills difficult. When diesel fuel enters surface water the petroleum hydrocarbon compound is believed to sit on top of the solution due to the greater density of water. The fuel breaks down in the water near the surface where one of the most toxic components within diesel fuel, the PAH's, become more bioavailable for aquatic biota and are excited in the presence of ultra violet light increasing the potential harm to aquatic life (Duggan 2013; Abdel-Shafy and Mansour 2016). Despite the belief that diesel initially remains on top of surface waters, CPW scientists have frequently observed the release of a sheen of oil or organics when walking on fine substrate. This occurred even long after a reach was tested by private and public entities and found to be below detection limits (P. Cadmus and M. May personal observations 2014-2021). Often this was observed immediately below a spill or too proximal to allow for the needed time to be dissolved. This suggests mixing of lotic environments may be greater than predicted in the above paradigm or suggests the surface area of the benthic zone is rapidly accumulating PAH's (Duggan 2013). The concentration, duration, loss and reactivity can be widely variable dependent on stream flows (volume, gradient, velocity), light exposure, sediment load, habitat structure and total volume of spilled toxicant. Challenges exist in measuring the true exposure of fish to diesel in the field. For the same reason, accurately dosing fish in laboratory toxicity trials is a barrier to devising effective standards, toxic thresholds or criteria to the protection of Colorado fish. Standardized methodologies for deriving species or mean acute threshold values has historically only considered mortality of organisms in acute toxicity experiments (Acute: first four days of exposure or first two days for some small organisms). The argument against consideration of sub-lethal endpoints is the organism in nature is sure to recover after the toxic exposure. It was assumed that changes in behavior, loss of olfactory function, reproduction, loss of fitness, up-regulation of genes, and any other sublethal endpoint has full likelihood to recover after the pulse stress event is ameliorated. Plains Minnow were exposed to sublethal levels of

diesel (75 mg/L nominal diesel fuel. 2.0 mg/L observed total petroleum hydrocarbons C10-C28) for twenty-four hours. In non-contaminated conditions we then conducted predator avoidance trials in which a Plains Minnow from a control group (0 mg/L diesel fuel) and exposure group (2.0 mg/L) competed to avoid predation by a Green Sunfish. Fathead Minnows were exposed to the same exposures and the avoidance of Yellow Perch, Green Sunfish and Creek Chub.

To explore the efficacy of shoaling (also known as "Schooling") we reproduced the experiment using schools of 4 Fathead Minnows exposed to diesel, 4 Fathead Minnows not exposed to diesel and assessed avoidance in the presence of multiple predators.

**METHODS**

Plains Minnow were obtained from the Colorado Parks and Wildlife Native Aquatic Species Restoration Facility (Alamosa, CO, USA). Fish were 35 mm +/-7 mm in standard length at the time of toxicant exposure. Fathead Minnow were obtained from Aquatic BioSystems (ABS Inc. Fort Collins, CO, USA) and were 35 ± 3 mm in length. Fish were marked with Visual Implant Elastomer (VIE) tags. These colors are visible by the naked eye with or without the florescence of an ultra-violet (UV) lamp. This allowed for identification of individuals during predator avoidance trials detailed below. Fish were exposed to MS-222 per label to anesthetize them during tagging. One of three colors was injected epidurally at the base of the dorsal fin. Fish were then promptly returned to a recovery tank after tagging and held for a minimum of four weeks. After four weeks no mortality from aestivation or tagging was observed in either species.

Exposures and predator avoidance studies using Plains Minnow occurred in 2020. Six liter stainless steel hotel pans served as exposure vessels. Each pan was filled with three liters of water that had been mixed to the appropriate concentration of diesel fuel to create nominal levels of 0, 75, and 150 mg/L fuel. A subsample of fish from each color (red, green, and blue VIE tag) group was then randomly assigned to each pan with a density of seven Plains Minnows (0.264 g +/- 0.24, 35 mm +/- 7) per vessel. Exposures were staggered temporally to allow for exactly twenty-four hours of exposure plus one hour recovery from exposure before examining the

predator avoidance. Each pan was aerated with an airline connected to a sodium silicate Pasteur pipette, possibly removing light volatile organics. Every six hours, a 50% static renewal of the water was conducted, extracting and replacing 1.5 liters of water mixed in order to the maintain the designated concentrations. A 16:8 hour light cycle using wide spectrum and ultraviolet UVA/UVB bulbs hung directly over the pans was used to simulate the natural UV regime. This has been found to photo-activate toxic compounds within petroleum but was orders of magnitude less intense than natural sunlight. Water samples were taken for assessment of total petroleum hydrocarbons C10-C28 by gas chromatograph immediately before and after the second six hour replenishment ("before" represents what is likely the lowest level of toxicant and "after" represents a fresh replenishment). Full mortality (100%) was observed after twenty-four hour exposure to the 150 mg/L nominal fuel concentration. After twenty-four hours of exposure, fish receiving 75 mg/L nominal fuel concentration showed no signs of morbidity, responded to visual stimulus, and vigorously attempted to escape palpation with a fine paint brush. Standardized toxicity trials following ASTM and EPA guidelines would characterize these fish as healthy and consider the nominal 75 mg/L concentration unable to illicit a toxic response.

Field collected Green Sunfish 80 mm to 150 mm in length were used for the predator species due to their larger gape limit. The native range of both predator and prey species co-occur in the Arkansas River in southeast Colorado. Predators were unfed for two days prior to running the predator avoidance trials.

Numerous circular black plastic 204 L tanks (KMB 101. Tuff Stuff Products, Terra Bella, California, USA. Figure D1) were used for predator avoidance trials. Each tank was 50% full of oxygenated reference (no toxicants) water at 14 -17° C. Technicians were hidden behind blackout curtains to limit disturbance or stress to fish. One exposed and one non-exposed Plains Minnow, of different color VIE tag, were assigned to a bottomless cylindrical stainless steel cage made of wire mesh (175 mm diameter) within the tub (Figure D2). This cylindrical cage prevented predation prior to the trial and allowed all organisms to acclimate to the tank for 30 minutes. A single Green Sunfish was then added to each tank and all fish acclimated an additional 30 minutes prior to starting each predator avoidance trial. The mesh cylindrical cage protecting Plains Minnow was lifted from the water column by pulling a cord from behind the

curtain. The fish within the tub were then censused every three minutes by careful examination from behind the curtains. If a fish had been eaten, the trial was stopped and both a UV and visible light were used to identify the surviving fish. Each surviving fish was then weighed, measured, and euthanized. Following each predation experiment trial, each vessel (tub) was fully drained, scrubbed, rinsed, and refilled before use in a subsequent predator avoidance trial. If no predation occurred after three hours the trial was ended, fish euthanized, not measured and not considered for statistical analysis of predator avoidance. In winter of 2021-2022, the same trial was conducted with Fathead Minnow evading Yellow Perch (collected from Seaman Reservoir, Larimer County, Colorado USA), Green Sunfish (Collected from Running Deer Open Space, Fort Collins, CO, USA ) and Creek Chub (collected from the South Platte River, south of Sterling Colorado; Table D1).

**RESULTS**

Observed toxicant exposure concentrations were well below the predicted or nominal concentrations. In-house assessments found most c10-c28 mass petroleum hydrocarbons were rapidly lost to the vessel or to volatilization. This was confirmed by an external (private sector) state and national certified analytical laboratory. This is a common problem for almost any laboratory conducting laboratory trials using static renewal techniques, especially petroleum hydrocarbons. For the nominal 150 mg/L diesel fuel exposure solutions, the hydrocarbons C10-C28 values immediately before and after the six hour replenishments averaged 15.55 (SD=1.7) mg/L and 7.3 (SD=1.06) mg/L total petroleum hydrocarbons C10-C28. Nominal treatment levels of 75 mg/L were observed at 2.0 mg/L (SD=0.7) total petroleum hydrocarbons C10-C28.

A total of twenty-three predator evasion trials were conducted. Three of the trials resulted in neither the exposed nor control fish being eaten after a total of three hours. These were excluded from statistical analysis. Of the twenty remaining successful trials, the diesel exposed Plains Minnow was eaten before the non-exposed Plains Minnow nineteen of the twenty times. This is significantly ($p = 0.00002$) greater than what would be expected (ten of twenty trials) if diesel fuel had no effect on predator avoidance. This trend continued when comparing

the evasion of Yellow Perch, Green Sunfish and Creek Chub by Fathead Minnow (Table D2). When four exposed Fathead Minnows and four non-exposed Fathead Minnows were allowed to form large schools before releasing predators, the targeting of prey that was previously exposed to diesel continued (Table D3).

## IMPLICATIONS

The results of the bioassay and the predator evasion trials indicate that even though the Plains Minnow appear unaffected and healthy by traditional standardized assessments, a twenty-four hour exposure to 2.0 mg/L of diesel fuel alters behavior important for survival well below the twenty-four hour toxic threshold (likely between 15.55 and 2.0 mg/L total petroleum hydrocarbons C10-C28). Acute standards and criteria have historically considered only lethal tests. During the formation of this policy, industry and regulatory agencies assumed that organisms will recover from all sublethal acute (four day or less) exposures. In this trial a sublethal compromise in behavior manifested into 95% mortality at a concentration that traditional acute toxicity trials would have considered a non-effect. Fish did not recover from the pollutant exposure despite removing the fish from the toxicant. Consumed fish did not recover from being consumed. As review of new chemical classes and the triannual (mandated every three years by Clean Water Act) review of existing standards are considered, inclusion of sublethal response variables for acute exposures should be considered if there is a chance that the measured function reduces survival in nature.

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**Figure D1:** The 204 L predator cage after initiating predator avoidance trial. (cage and air stone was removed for photo).

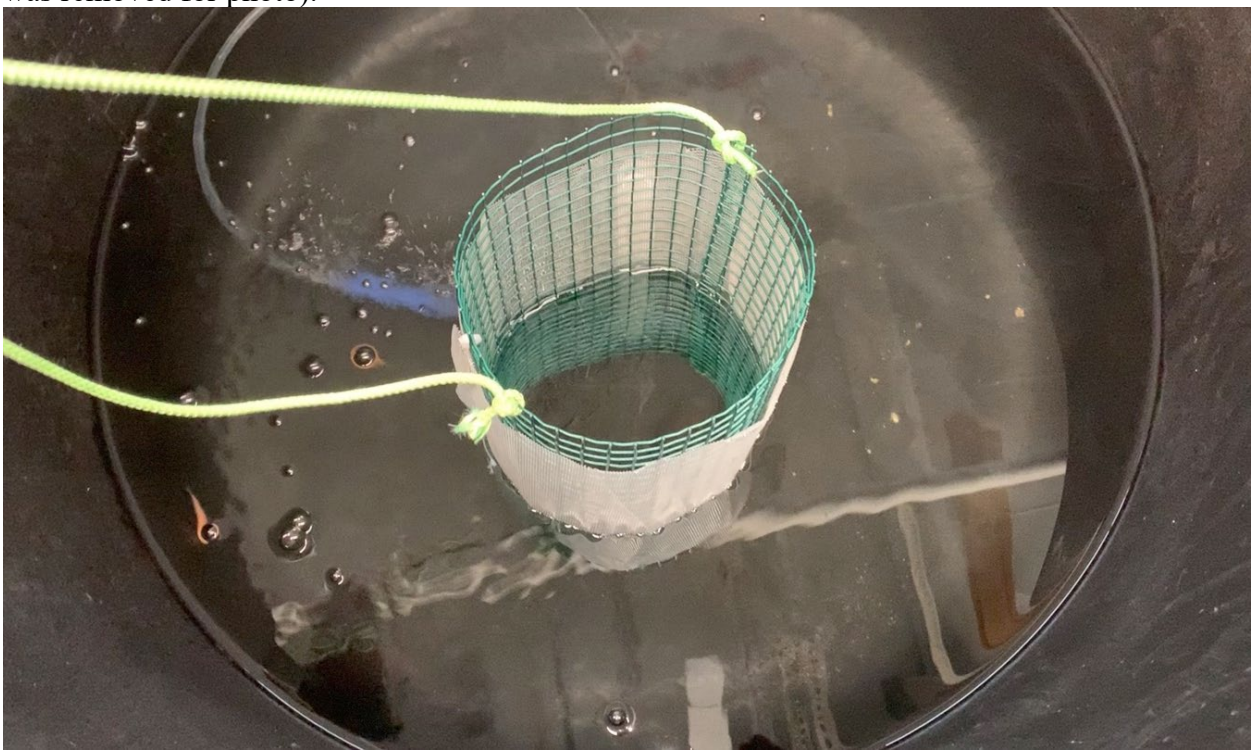

**Figure D2:** The 204 L predator cage with wire/mesh 175 mm diameter inner cage. Attached cord allows trial to be started by technician from behind curtains.

Table D1: Fish Lengths of Prey and Preditor Species

| Fish Species | Size (mm ± SD) |
|---|---|
| Plains Minnow | 40 ± 12 |
| Fathead Minnow | 35 ± 3 |
| Flathead Chub | 44 ± 15 |
| Green Sunfish | 160 ± 38 |
| Creek Chub | 170 ± 25 |
| Yellow Perch | 200 ± 20 |
| Brown Trout | 180 ± 17 |

Table D2: Predator Evasion Trial Results within Three Hours of Diesel Exposure. Proportion of trials in which fish exposed to diesel or not exposed to diesel were consumed by a predator after sublethal, sub-acute diesel exposure. Effects on Predator Evasion. These trials included only one exposed prey fish and one control fish in each trial.

| Predator Species /Prey Species | Total Mortality Rate For Exposed Fish | Total Mortality Rate For Control Fish | Total number of Trials |
|---|---|---|---|
| **Green Sunfish / Plains Minnow** | 0.857 | 0.143 | 7 |
| **Yellow Perch / Fathead Minnow** | 0.857 | 0.143 | 7 |
| **Green Sunfish / Fathead Minnow** | 1.000 | 0.000 | 12 |
| **Creek Chub / Fathead Minnow** | 0.667 | 0.333 | 16 |

Table D3: Predator Evasion Trial Results for Large Schools of Prey within Three Hours of Diesel Exposure. Proportion of trials in which fish exposed to diesel or not exposed to diesel were consumed by predators when in large schools. These trials included four exposed prey fish and four control fish in each trial.

| **Predator Species /Prey Species** | **Total Mortality Rate For Exposed Fish** | **Total Mortality Rate For Control Fish** | **Total number of Trials** |
|---|---|---|---|
| Green Sunfish / Flathead Chub | 0.75 | 0.375 | 2 |
| Green Sunfish / Fathead Minnow | 0.783 | 0.087 | 6 |