Water Pollution Studies

Pete Cadmus, Ph.D.
Aquatic Research Scientist/Toxicologist

Tawni B. R. Firestone, Ph.D.
Aquatic Research Scientist, Aquatic Toxicology and Disease

Darian Woller
Colorado State University, Graduate Student

Riley Dils
Colorado State University, Research Associate

Annual Report

Colorado Parks & Wildlife

Aquatic Research Section

Fort Collins, Colorado

November 2024

STATE OF COLORADO

Jared Polis, Governor

COLORADO DEPARTMENT OF NATURAL RESOURCES

Dan Gibbs, Executive Director

COLORADO PARKS & WILDLIFE

Jeff Davis, Director

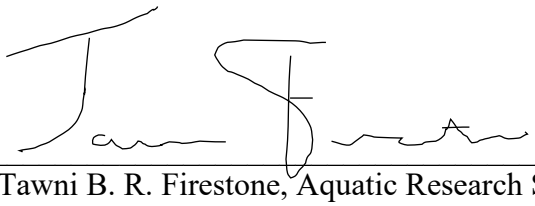
WILDLIFE COMMISSION

Dallas May, Chair
Richard Reading, Vice Chair
Karen Bailey, Secretary
Jessica Beaulieu
Marie Haskett
Jack Murphy
Gabriel Otero
Murphy Robinson
Tai Jacober
James Jay Tutchton
Eden Vardy

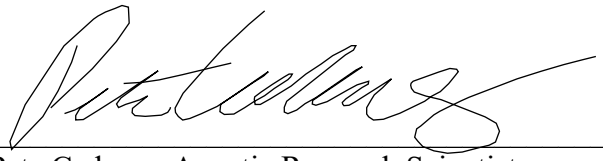
Ex Officio/Non-Voting Members: Kate Greenberg, Dan Gibbs

AQUATIC RESEARCH STAFF

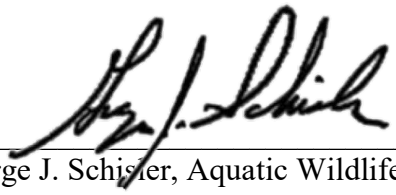
George J. Schisler, Aquatic Research Leader
Brian W. Avila, Aquatic Research Scientist, Population Genetics
Peter J. Cadmus, Aquatic Research Scientist/Toxicologist, Water Pollution Studies
Kelly Carlson, Aquatic Research Program Assistant
Eric R. Fetherman, Aquatic Research Scientist, Salmonid Disease Studies
Tawni B. R. Firestone, Aquatic Research Scientist, Aquatic Toxicology and Disease
Ryan M. Fitzpatrick, Aquatic Research Scientist, Eastern Plains Native Fishes
Adam G. Hansen, Aquatic Research Scientist, Coldwater Lakes and Reservoirs
Zachary Hooley-Underwood, Aquatic Research Scientist, West Slope Three-Species Studies
Matthew C. Kondratieff, Aquatic Research Scientist, Stream Habitat Restoration
Dan A. Kowalski, Aquatic Research Scientist, Stream and River Ecology
Jesse M. Lepak, Aquatic Research Scientist, Warm and Coolwater Lakes and Reservoirs
Eric E. Richer, Aquatic Research Scientist/Hydrologist, Stream Habitat Restoration
Kevin B. Rogers, Aquatic Research Scientist, Cutthroat Trout Studies
Andrew J. Treble, Aquatic Database Manager, Aquatic Data Management and Analysis
Brad Neuschwanger, Hatchery Manager, Fish Research Hatchery
Tracy Davis, Hatchery Technician, Fish Research Hatchery
Troy Meyer, Hatchery Technician, Fish Research Hatchery
Karen Hertel, Librarian

Prepared by: 

Tawni B. R. Firestone, Aquatic Research Scientist

Prepared by: 

Pete Cadmus, Aquatic Research Scientist

Approved by: 

George J. Schisler, Aquatic Wildlife Research Chief

Date: 11/11/2024

NEEDS AND OBJECTIVES OF THE TOXICOLOGY LABORATORY

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 estradiol 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 with fish, aquatic macroinvertebrates, algae and other fish forage species.
2. Improve state and national water quality standards to ensure they are protective of aquatic life of Colorado. These standards include toxicants (*e.g.* Fe, Se, Cu, Cd, Zn, Al, Mn, benzene, petrochemicals, and pharmaceuticals) and physical properties (*e.g.* total suspended solids, temperature, and 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, and winter survival), consider phenology, species interaction, multi-generational effects, and exposure regimes unique to Colorado.
    c. Consider ecologically relevant sub lethal 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, and cortisol measurements).
    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).
    g. 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 a specialized set of skills 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.

References

Bravo, C. F., L. R. Curtis, M. S. Myers, J. P. Meador, L. L. Johnson, J. Buzitis, T. K. Collier, J. D. Morrow, C. A. Laetz, F. J. Loge, and M. R. Arkoosh. 2011. Biomarker responses and disease susceptibility in juvenile rainbow trout *Oncorhynchus mykiss* fed a high molecular weight PAH mixture. Environmental Toxicology and Chemistry 30:704-714.

Clements, W. H., D. M. Carlisle, J. M. Lazorchak, and P. C. Johnson. 2000. Heavy metals structure benthic communities in Colorado mountain streams. Ecological Applications 10:626-38.

Clements, W. H., J. T. Oris, and T. E. Wissing. 1994. Accumulation and food-chain transfer of floranthene and benzo[a]pyrene in *Chironomus riparius* and *Lepomis macrochirus*. Archives of Environmental Contamination and Toxicology 26:261-266.

Colorado Department of Public Health and Environment – Water Quality Control Commission. 2013 Regulation No. 31. The basic standards and methodologies for surface water (5 CCR 1002-31)

Damasio, J. B., C. Barata, A. Munne, A. Ginebreda, H. Guasch, S. Sabater, J. Caixach, and C. Porte. 2007. Comparing the response of biochemical indicators (biomarkers) and biological indices to diagnose the ecological impact of an oil spillage in a Mediterranean River (NE Catalunya, Spain). Chemosphere 66:1206-1216.

Daughton, C. G. 2004. Non-regulated water contaminants: Emerging research. Environmental Impact Assessment Review 24:711-732.

General Accounting Office. 1994. Toxic Substances Control Act: preliminary observations on legislative changes to make TSCA more effective (Testimony, 07/13/94, GAO/T-RCED-94-263)

Giesy, J. P., S. M. Bartell, P. F. Landrum, G. J. Leversee, and J. W. Bowling. 1983. Fates and biological effects of polycyclic aromatic hydrocarbons in aquatic systems. United States Environmental Protection Agency, Athens, Georgia, USA. EPA 600-S3-83-053.

Lamoureux, E. M., and B. J. Brownawell. 1999. Chemical and biological availability of sediment-sorbed hydrophobic organic contaminants. Environmental Toxicology and Chemistry 18:1733-1741.

Lytle, D. A., and B. L. Peckarsky. 2001. Spatial and temporal impacts of a diesel fuel spill on stream invertebrates. Freshwater Biology 46:693-704.

Mineral Policy Center. 1997. Golden dreams, poisoned streams. Washington, D.C., USA

Neff, J. M. 1979. Polycyclic aromatic hydrocarbons in the aquatic environment: sources, fates, and biological effects. Applied Science Publishers Ltd, London, UK.

Schuler, L. J., M. Wheeler, A. J. Bailer, and M. J. Lydy. 2003. Toxicokinetics of sediment-sorbed benzo[a]pyrene and hexachlorobiphenyl using the freshwater invertebrates *Hyalella azteca*, *Chironomus tentans*, and *Lumbriculus variegates*. Environmental Toxicology and Chemistry 22:439-449.

Stephen, C. E., D. I. Mount, D. J. Hansen, J. R. Gentile, G. A. Chapman, and W. A. Brungs. 1985 Guidelines for deriving numerical national water quality criteria for the protection of aquatic organisms and their uses. US Environmental Protection Agency. Washington, DC USA. PB85-227049

USDA Forest Service. 1993. Acid mine drainage form impact of hard rock mining on the National Forests: A management challenge. Program Aid 1505.

Application Methods to Apply EarthTec QZ® in an Attempt to Eradicate Zebra Mussels from Highline Lake

Zebra Mussels *Dreissena polymorpha* are a non-native, invasive mussel to Colorado and are known to have significant ecological, economic, and recreational impacts among water bodies if a population of the mussel is established. On September 14, 2022 the Aquatic Nuisance Species (ANS) Sampling and Monitoring Team found an adult Zebra Mussel located on an artificial substrate located in Highline Reservoir (Loma, CO). Nine additional adult Zebra Mussels were found on a boat and among boat ramps in the reservoir in October and two adults were found in Mack Wash below at the effluent of the reservoir in November, 2022. CPW has previously adopted the Western Regional Panel's Building Consensus guidelines for classifying water bodies for mussel detections. As such, Highline Reservoir has been determined as infested for Zebra Mussels due to a reproducing and recruiting established population. In an attempt to eradicate the mussel from Highline Reservoir the lake was lowered approximately 30 feet and a team of biologists and researchers applied Earthtec QZ®, a copper-based, EPA-registered molluscicide, at 4 ppm over three treatments to the lake. Water samples were collected throughout the treatment period and as the lake filled to understand total concentration of copper.

Assessing Water Quality Impacts on Genetic Recruitment of Native Suckers in the Gunnison River

The Bluehead Sucker and Flannelmouth Sucker are native to the Colorado River but currently occupy less than 50% of their historic ranges due to hybridization with non-native species. Native sucker primarily spawn in tributaries, presenting opportunities for targeted management to reduce hybrid encounters during reproduction. This study investigates the role of water quality in tributaries on genetic recruitment into the Gunnison River, focusing on strategies to mitigate hybridization risks. We will evaluate tributary networks through a five-year analysis of shallow genetic divergence among spawning populations using high-throughput genotyping methods. This approach aims to identify recruitment bottlenecks associated with thermal and water quality transitions from warmer tributary habitats to the cooler, regulated mainstem Gunnison River. Additionally, movement data from PIT-tagged adults will confirm tributary spawning participation, complementing genetic insights. Our objectives include establishing a genetic marker panel for assessing population structure, identifying productive tributaries, and implementing a GSI based tagging approach to estimate recruitment and survival as a function of water quality. Fish collection for this project began in May 2024.

Effects of Chloride on Eastern Plains Fish

Traditional toxicity tests were conducted using field collected Western Mosquitofish (*Gambusia affinis*) and Plains Topminnow (*Fundulus sciadicus*) to examine tolerance to $CaCl_2$. Resistance to acute thermal events (Critical Thermal Maxima) was assessed for both species with and without exposure to $CaCl_2$.

Effects of Copper, Chloride, Sulfate, and Chlorine on Drift and Chemical Avoidance of Benthic Macroinvertebrates and Salmonids

Experiments to examine chemical avoidance behavior of fish and aquatic invertebrates are ongoing. Analytes include Chloride, Sulfate and Chlorine. Novel methods were employed to examine invertebrate drift after exposure to copper.

Effects of Road Salts on Cutthroat Trout

A series of acute road salt toxicity trials were conducted on Cutthroat Trout *Oncorhynchus clarkii* to determine if road salts and fish density influence survival, thermal tolerance, or stress levels of the fish. The results in this report suggest that various concentrations up to 860 mg L^{-1} of $MgCl_2$ do not cause a physiological effect on stress levels in one-year-old Cutthroat Trout. However, we did detect a difference in cortisol concentrations when densities of fish are high.

Establishing Temperature Tolerance Ranges for Native Fish Species through Electrocardiogram Analysis

Water temperature is a critical abiotic factor influencing fish survival, yet many species struggle to adapt to extreme and rapid temperature fluctuations. In Colorado, native fish populations are exposed to these conditions, necessitating the assessment of their temperature tolerance, particularly in adult stages. This study aims to establish protective temperature standards for adult Bluehead Sucker, Flannelmouth Sucker and Roundtail Chub using acute laboratory tests, focusing on physiological endpoints rather than traditional larval tests. We measure cardiac output using electrocardiograms (ECGs) while gradually increasing water temperatures at a rate of +0.3°C min^{-1} until cardiac arrhythmia is detected. This approach allows us to identify the thermal tolerance ranges critical for the species' survival. The findings of this study will contribute to developing field-based temperature criteria that align with Colorado Water Quality Control Commission Policy 06-1. By understanding the physiological response of adult fish to temperature changes, we can better inform conservation strategies and water management practices aimed at protecting native species. The first step of this project was to optimize our anesthetizing and ECG protocol using Rainbow Trout and White Suckers, included in this report.

Field-based Temperature Standards for Bluehead Sucker (*Catostomus discobolus*), Flannelmouth Sucker (*Catostomus latipinnis*), and Roundtail Chub (*Gila robusta*)
(Collaborative project with Colorado State University)

Water temperature is one of the most important abiotic factors that contribute to larval fish survival and many fish species are poorly adapted to survive variable water temperatures that lie outside normal annual or seasonal changes. Bluehead Sucker *C. discobolus*, Flannelmouth Sucker *C. latipinnis*, and Roundtail Chub *Gila robusta* are endemic to western Colorado and use intermittent and ephemeral streams for refugia from high main-stem flows, foraging, and for larval rearing. A range-wide conservation agreement and strategy for the three species has been prepared for the Colorado River Fish and Wildlife Council throughout their respective ranges with a collaborative effort between agencies to implement conservation measures. Despite the conservation agreement, the impact of changing temperatures on larval stages of the three species has received little attention. One study attempted to define thermal ranges for larval Bluehead Sucker, but this was based in a laboratory and did not account for field stream temperature variation or the possibility of acclimation or adaptation of local wild fish

populations. To address the potential stressors or mechanisms underlying the upper and lower temperature tolerance of larval three species, we have started to outline a project in collaboration with Colorado State University to conduct stream-side UILT, LILT, CTMax, and CTMin tests with larval fish collected from various populations of the three species.

Infrastructure Improvements, Method Development, and Experiments to Ameliorate the Negative Effects of Iron and Iron Oxidizing Bacteria at Poudre River State Trout Hatchery

Water from wells in pyritic parent material often contains high levels of ferrous iron. A CPW Hatchery has long been plagued by iron oxidizing bacteria that occludes gill surfaces. Aquatic Toxicology Laboratory Staff aided hatchery staff in infrastructure improvements, equipment fabrication, and experimentation. Experiments were not limited to: constructed air ducts throughout Poudre River State Trout Hatchery, PR1 and PR2 isolation units, installed numerous blowers, constructed an array of aquaria for experiments, constructed a modular water conditioning system, fabricated hatchery specific aquaculture tools and infrastructure (flow meters, egg rearing baskets, supply lines), and trained hatchery staff in chemical sampling methods. Three series of experiments explored use of aeration and reaction times to oxidize ferrous iron to ferric iron and use of non-specific antibiotic treatments to reduce iron oxidizing bacteria.

Laboratory Infrastructure Maintenance

Extensive repair of well service lines and typical repair and preventative maintenance was conducted. This is not limited to the following systems: Replacement/rerouting of high hardness water well supply plumbing to both the primary laboratory and the auxiliary laboratory, replacement of broken or unserviceable high security locks, restoration of laboratory space and buildings, HVAC (hood and blower) maintenance and repair, hazardous material disposal, removal of obsolete infrastructure and instruments, replacement and rebuilding of laboratory specific pumps, and servicing of equipment.

Pathogenesis of *Renibacterium salmoninarum* in Chinook Salmon following intraperitoneal injection: Description of disease progression using qPCR and histopathology

Renibacterium salmoninarum, the cause of bacterial kidney disease (BKD), impacts salmonid populations. Much of our understanding of the pathology in salmonids comes from evaluating fatal infections in wild populations, hatchery stocks, or experiments that are typically focused on tissue damage and immune response. The progression of BKD throughout is not well represented in the literature and is important to detect and diagnose fish disease. To evaluate the progression of disease, we injected *R. salmoninarum* intraperitoneally into one-year-old Chinook Salmon. We sampled kidney, liver, spleen, heart, brain, and gills for histopathology, and kidney and liver tissues for quantitative PCR over ten weeks. Gram stains first detected Gram-positive bacteria in the perisplenic adipose at week one, then the liver at week four, and kidney at week five. Results from qPCR revealed increasing bacterial loads in kidney and liver over the course of the study. Bacteria appeared with qPCR analysis only after one week in spleen tissue and five weeks post-injection in the kidney tissue, corroborating histologic evidence of increasing organ damage and bacterial burden as the infection progresses. Our results indicate BKD is a systemic disease, and diagnostic testing of liver or spleen with perisplenic adipose may aid in early detection.

The Importance of a Nuanced Approach to Developing Aquatic Species-specific Temperature Standards: A Review

The regulation and protection of aquatic organisms has long been a critical environmental concern, with temperature standards playing a pivotal role in ensuring their wellbeing. In the past, policymakers and resource managers have relied heavily on simplistic water temperature metrics, such as maximum allowable limits, to safeguard aquatic species. While these broad guidelines provided a baseline, they often fell short in accounting for the dynamic and heterogeneous nature of aquatic habitats, where organisms may encounter a diverse range of thermal conditions over time and space. The shortcomings of this approach became increasingly evident as climate change and other anthropogenic stressors began to reshape aquatic environments, exposing the limitations of one-size-fits-all temperature standards. Today, there is a growing recognition that a more nuanced and scientifically-grounded approach is necessary to effectively protect aquatic organisms. Integrating laboratory-based thermal tolerance studies into field-based assessments can provide a deeper understanding of species-specific responses to temperature fluctuations, allowing for the development of more targeted and adaptive management strategies. By simulating a range of thermal conditions in controlled settings, researchers can elucidate the physiological thresholds, behavioral adaptations, and potential acclimation abilities of various aquatic species. By bridging the gap between controlled experiments and real-world conditions, resource managers and policymakers will be better equipped to develop robust temperature standards.

TECHNICAL SUPPORT

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.

Biomonitoring Consulting and Annual Service

Benthic macroinvertebrate sampling (semi-annual) and fish sampling (annual) was conducted at North Fork of Clear Creek water basin (Blackhawk, CO, USA) where a mine effluent treatment plant was installed by state and federal entities, and where proposed water diversions, retention, and off basin attainment is being proposed. Site visit and development of sampling methods for aquatic macroinvertebrates in Bear Creek (El Paso and Teller County Colorado) is ongoing.

Hatchery Water Quality Testing

A state-wide hatchery water quality testing at all production water sources was implemented for two years starting in spring 2023. This project is a collaborative effort between CPW's Toxicology Laboratory, aquatic and hatchery section, and the River Watch Laboratory. The mission of this testing is to monitor water quality and other indicators of fish health at a Colorado hatchery and utilize this data to inform hatchery manager about the condition of their production water sources.

Water samples for metal analysis were collected from each of the Colorado hatcheries during their high flow spring seasons. Water samples were collected by hatchery employees from each of the water sources on the unit. Water samples were passed through a 0.45 µm filter (filtered sample) and a syringe without a filter (nonfiltered sample) and preserved with high purity nitric acid to a pH of <2. Metals were measured using an Inductively Coupled Plasma (ICP) spectrometer coupled prior to each use and the calibration was verified using NIST traceable QAQC standards. Duplicates and spikes were included in each run for QAQC. All samples were analyzed within 6 months of receiving the samples.

The Colorado Department of Public Health and Environment (CDPHE) has set acute and chronic standards to protect aquatic life for the following metals analyzed in the Aquatic Toxicology and River Watch Laboratory: aluminum (Al), arsenic (As), cadmium (Cd), copper, (Cu), iron (Fe), manganese (Mn), lead (Pb), selenium (Se), and zinc (Zn). Acute and chronic compliance standards, and water hardness were listed in each hatchery report for specific site results. Hardness was calculated for each site based on the measured calcium (Ca) and magnesium (Mg) values and were also included in each report. Nonregulated elements listed in each report included calcium (Ca), potassium (K), magnesium (Mg), and sodium (Na). 2019 CDPHE criteria equations, which for many metals are hardness dependent, were also included in each hatchery report for reference.

A total of 25 water quality data hatchery reports have been analyzed in FY24 and reported to the hatchery chief to disseminate to hatchery managers. These reports were also shared with the Water Quality Sections and the Aquatic Animal Health Laboratory.

Invertebrate and Fish Sample Processing

Archived samples from field biomonitoring and experimental insect samples were picked and identified. These sites include monitoring of mine effluent mitigation efforts, potential native fish reintroduction sites, and hazmat spills. Fish from numerous fish kills and poor performing management areas were dissected and tissue was examined as requested.

Milt Extender Production

Milt extender was produced for federal and state natural resource management agencies across the country March of 2024.

Reclamation Projects and Rotenone Analysis

The CPW Aquatic Toxicology Laboratory conducted on-site assessment of rotenone during chemical reclamation projects to restore native Cutthroat Trout populations. The CPW Aquatic Toxicology Laboratory provided on-site assessment at the following projects: Rito Hondo Reclamation Project (Creede, CO: August 2023), George Creek Reclamation Project (Red Feather, CO: August 2023), Lost Dog Creek Reclamation Project (Steamboat, CO: August 2023), and Hunt Basin Reclamation Project (Sangre de Cristo Wilderness Area, CO: September 2023).

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.

- Lepak, J. M., W. M. Pate, **P. Cadmus**, A. G. Hansen, K. D. Gallaher, and D. B. Silver. 2024. Response of an invasive aquatic crustacean to the fish toxicant rotenone. *Lake and Reservoir Management* 40(3): 330-337.
- **Riepe, T. B.,** Z. E. Hooley-Underwood, and M. Johnson. 2024. Temperature tolerance of larval flannelmouth sucker acclimated to three temperatures. *Fishes* 9:181.

Internal presentations to CPW staff were used to update managers on current research and inform management decisions in Colorado. One presentation was given at the CPW Aquatic Hatchery Meeting.

- **Riepe, T. B.** 2023. What's in your water samples? Colorado Parks and Wildlife Hatchery Manager's Meeting. Vail, Colorado. September 13, 2023.

External presentations provided an opportunity to give research updates to managers both within and outside Colorado. Three talks were given at the Colorado/Wyoming Chapter of the American Fisheries Society meeting and one was given at the Range-wide 3 species conservation team meeting.

- **Riepe, T. B.** 2023. Bluehead Sucker and Flannelmouth Sucker Temperature Research Update. Range-wide 3-Species Conservation Team Meeting. Virtual. November 13, 2023.
- Lewis S., Y. Kanno and **P. Cadmus.** 2024. "An ecotoxicological evaluation of salinity on lethal and sub-lethal effects in invasive mosquitofish and native plains topminnow" Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, WY. February 28, 2024.
- **Riepe, T. B.** and Z. E. Hooley-Underwood. 2024. Temperature tolerance of Flannelmouth Sucker larvae acclimated to three temperatures. Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, WY. February 29, 2024.
- Dils R., **T. B. Riepe**, P. Schaffer, D. L. Winkelman, and E. R. Fetherman. 2024. Pathogenesis of *Renibacterium salmoninarum* in Chinook Salmon following intraperitoneal injection: Description of disease progression by qPCR and histopathology. Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, WY. February 29, 2024.

- McDevitt R., **T. B. Riepe**, P. Schaffer, C. Wells, E. R. Fetherman, and D. L. Winkelman. 2024. The susceptibility of Chinook Salmon to two strains of *Renibacterium salmoninarum*. Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, Wyoming. February 29, 2024.
- Clements, W.H., **P. Cadmus**, C. Kotalik. 2024. "Quantifying the relative effectiveness of remediation and restoration treatments in the Upper Arkansas River, Colorado" Rocky Mountain chapter of the Society of Environmental Toxicology and Chemistry. Laporte, CO. April 17, 2024.
- Schaffer P.A., **P. Cadmus**. 2024. "What's wrong with this fish?" Poster. Rocky Mountain chapter of the Society of Environmental Toxicology and Chemistry. Laporte, CO. April 17, 2024.
- Lewis S., Y. Kanno and **P. Cadmus**. 2024. "An ecotoxicological evaluation of salinity on lethal and sub-lethal effects in invasive mosquitofish and native plains topminnow" Rocky Mountain chapter of the Society of Environmental Toxicology and Chemistry. Laporte, CO. April 17, 2024.
- Dils R., and **T. B. Riepe**. 2024. On the pulse: Investigating fish temperature tolerance. Colorado State University, Celebrate Undergraduate Research and Creativity Showcase. Poster Presentation. Fort Collins, CO. April 18, 2024.

In addition to public and professional meeting presentations, one presentation was given at a wildlife disease ecology class at Colorado State University. During this class students learned about various diseases and toxicants that can negatively affect fish in aquaculture facilities or in wild populations.

- **Riepe, T. B.** Guest lecturer: Diseases and Toxicants Effecting Fish Health. FW467/567 – Wildlife Disease Ecology. Colorado State University: Fish, Wildlife, and Conservation Biology. October 24, 2023.
- **Riepe, T. B.** Guest lecturer: Diseases and Toxicants in Aquaculture. FW467/567 – Wildlife Disease Ecology. Colorado State University: Fish, Wildlife, and Conservation Biology. October 24, 2023.
- **Cadmus, P.** 2024 Guest lecturer: Introduction to Ecotoxicology. FW 544 – Ecotoxicology. Colorado State University: Fish, Wildlife, and Conservation Biology. January 23, 2024.
- **Cadmus, P.** 2024 Guest lecturer: Factors that modify toxicity. FW 544 – Ecotoxicology. Colorado State University: Fish, Wildlife, and Conservation Biology. January 25, 2024.
- **Cadmus, P.** 2024 Guest lecturer: Aquatic toxicology experiments and how they inform policy. FW 544 – Ecotoxicology. Colorado State University: Fish, Wildlife, and Conservation Biology. January 30, 2024.
- **Cadmus, P** Short Course: Algal Sampling Methods for Lentic and Lotic Ecosystems" Rocky Mountain Chapter of the Society of Environmental Toxicology and Chemistry. Laporte, CO. April 18, 2024.

Application Methods to Apply EartTec QZ ® in an Attempt to Eradicate Zebra Mussels from Highline Lake

Dr. Tawni B. R. Firestone

This project is a collaborative effort with many CPW personnel including Robert Walters (Invasive Species Specialist), Ben Felt (Senior Aquatic Biologist), Tyler Swarr (Conservation Biologist), Kristina Morben (Aquatic Biologist), Melynda May (Water Quality Specialist), Megan McConville (Water Quality Analyst), Maddie Baker (Invasive Species Technician), and help from many biologists, park managers, and aquatic technicians.

1.1 Introduction

Invasive species pose high ecological threats to native species in infested water bodies including displacing the native species, influence on food web structures, alternating ecosystem processes, altering water chemistry, and invading water infrastructure. Zebra Mussels *Dreissena polymorpha* are small freshwater bivalves native to the Black, Caspian and Aral Seas (Ludyanskiy et al. 1993) and were introduced to North America in 1886 (Herbert et al. 1989). They have since colonized many freshwater ecosystems in 33 states and across southern Canada (Stokstad 2007; Wimbush et al. 2009). Spread and colonization are primarily driven by recreation in areas with heavy boat use (Frischer et al. 2005). Since most of the mussels are typically found near marinas it is likely that many mussel infestations occur because the adult mussel attaches to watercraft. Additionally, the larvae can also be transported in boat water reservoirs including live wells, ballast water, and bilges and the adults can survive out of water for up to 30 days.

Zebra Mussels often have little competition with any native fish or mussel species, predators, or parasites in North America (Casagrandi et al. 2007). Thus, the infestation of a population is mostly dictated by water quality and high reproductive success of the organism. Water bodies that support Zebra Mussel populations have occurred when annual water temperatures are less than 30°C, when nutrients support photosynthetic biomass growth, salinity does not exceed 3 ppt, pH is greater than 7.4, and when calcium concentrations are greater than 12 mg L^{-1} (Morton 1971; Ten and Davids 1982; Spring and Rose 1988; Kovalak 1989; Smirnova and Vinogradov 1990; O'Neill and MacNeill 1991; Sprung 1995; Wimbush et al. 2009). Once the mussels are established, high population level survival is supported by their high reproductive success. Mussels broadcast spawn facilitating large dispersal of the larvae throughout the water body. Previous research has described the restrictions of the expansion of the Zebra Mussel is only due the limits of their own biological needs, which are not very selective.

Control methods to reduce threats of Zebra Mussels have been adopted in many states through aquatic nuisance species (ANS) programs. Prevention and education is often the first line of defense and watercraft inspection and decontamination programs are often put into practice.

Colorado has implemented a "Clean – Drain – Dry" method for all watercraft. This method includes cleaning all waders, boots, boats, trailers, vehicle hitches, and motors to remove any plants, animals, and mud; completely draining any water reservoir from a watercraft (live wells, bait containers, ballast and bilge tanks, and engine cooling systems); and allowing sufficient time for all watercraft and equipment to completely dry before launching into another body of water. Despite these prevention measures, Zebra Mussels were detected for the first time in one water body in western Colorado.

In the fall of 2022, Zebra Mussels were discovered in Highline Lake, a high recreational-use Lake in Loma, CO. On September 14, 2022 the ANS Sampling and Monitoring Team found an adult Zebra Mussel located on an artificial substrate located in Highline Lake. Nine additional adult Zebra Mussels were found on a boat and among boat ramps in the reservoir in October and two adults were found in Mack Wash below at the effluent of the lake in November, 2022. CPW has previously adopted the Western Regional Panel's Building Consensus guidelines for classifying water bodies for mussel detections. As such, Highline Lake has been determined as infested for Zebra Mussels due a reproducing and established population, thus we attempted to use a copper-based treatment to eradicate the mussels.

Earthtec QZ® is a copper-based algaecide marketed to control Dreissenid mussels. The active ingredient is a formulation of copper sulfate pentahydrate combined with a base acid and is delivered in the cupric ion form to make it more biologically available and less likely to precipitate out of the water column (Hammond and Ferris 2019). Cupric ions are known to disrupt normal functions of the body, making Cu^{2+} lethal to almost all aquatic life forms (Watters et al. 2013). As such, Earthtec QZ® has been successfully used to eradicate Quagga and Zebra Mussels in laboratory experiments and in small bodies of water in part due to the toxic effect on gastropod embryos and larvae (Watters et al. 2013; Iwanyckyj et al. 2017; Caromosini et al. 2018; Hammond and Ferris 2019). However to our knowledge, the size and magnitude of an eradication project as large as Highline Lake has not been attempted.

In an attempt to eradicate the mussel from Highline Lake, the lake was lowered approximately 30 feet and a team of biologists and researchers applied Earthtec QZ® at 4 ppm over three treatments to the lake. Water samples were collected throughout the treatment period and as the lake filled to understand total concentration of copper and proper mixing throughout the lake. This report describes the application methods, water sample analysis, and a modeling method to understand overall copper concentrations across the reservoir during the treatment.

1.2 Methods

Highline Lake is a 135 acre lake, with a max depth of 62 feet, and is a surface water fed lake at 4,693 feet in elevation. It is part of the Colorado River Drainage where the effluent drains into Mack Wash and to the Colorado River roughly 6 miles downstream (Figure 1.1). The lake substrate is primarily sand and supports aquatic plant growth and warm water fish species including Smallmouth Bass *Micropterus dolomieu*, Common Carp *Cyprinus carpio*, Black Crappie *Pomoxius nigromaculatus*, Black Bullhead *Ameiurus melas*, and White Sucker *Catostomus commersonii*. The lake is open to watercraft during the boating season (March – September) and can see upwards of ~2,800 boats per season (CPW Internal Reports). A

prevention program, including watercraft decontamination areas, was implemented for early-detection monitoring. The ANS program annually conducted baseline assessments of aquatic invasive species, using plankton tows for veligers, snorkel surveys, and assessments of plant species. No Zebra Mussels were found in Highline Lake, nor in any Colorado water body, prior to September 2022.

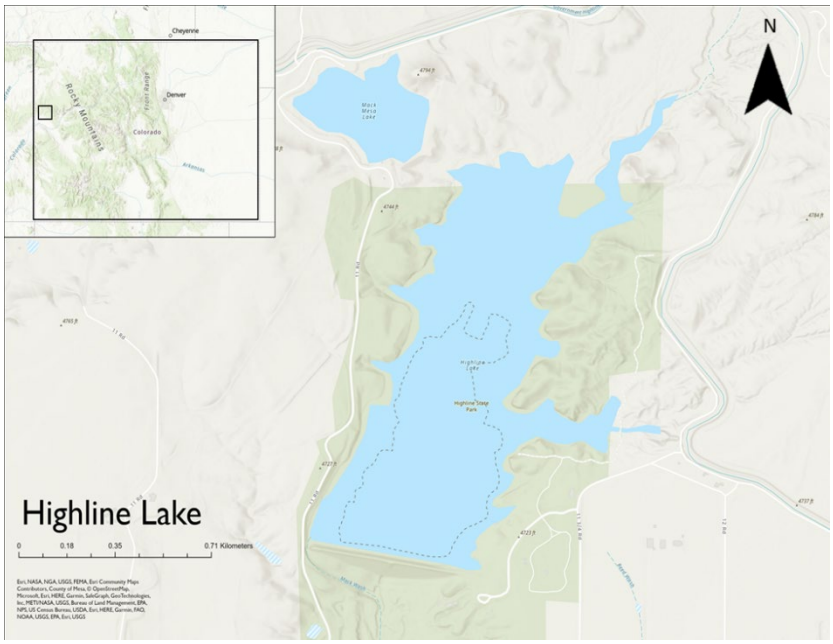


Figure 1.1 Highline Lake located in Loma, Colorado at full capacity (blue polygon outline) and after draining the reservoir prior to the copper application (dotted line).

1.2.1 *Bathymetry*

Prior to the EarthTec QZ® application, Highline Lake was drained to about 25% capacity (approximately 30 ft below maximum capacity; Figure 1.1). After the lake was lowered we collected bathymetry data points to determine the depth and estimate the volume of water to determine the correct EarthTec QZ® dosage. Measuring the spatial changes of the lake was critical to understand the dilution of copper throughout the treatment period. Bathymetry points were collected over three time periods; prior to the treatment, during, and after the lake was refilled. Data points were collected via a raft and Lowrance sonar unit. Depths were collected over various points and GPS points were documented. The shoreline points were collected from the front of the raft because the shoreline was too muddy to walk. Once the survey was completed, data was uploaded in to the ArcPro software to create the bathymetry map. The initial bathymetric measurements, prior to treatment, are included in this report (Figure 1.2)

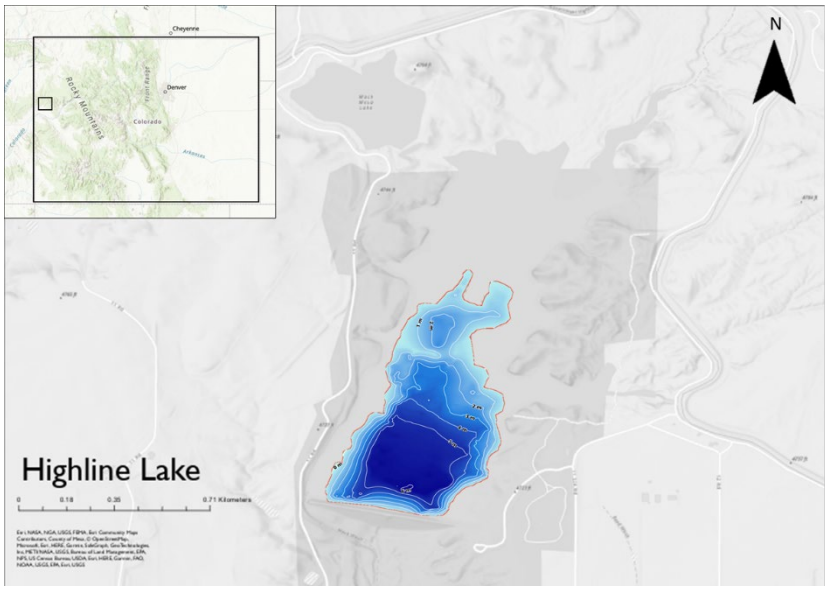


Figure 1.2 Bathymetry measurements prior to the first copper treatment and after the lake water level was lowered.

1.2.2 *Safety Precautions*

Prior to applying the EarthTec QZ®, all personnel were briefed on project safety and first aid measures in the event of exposure to the eyes, skin, or ingestion of the chemical. All personnel were provided with a Tyvek suit, face shield or goggles, an N-95 mask and chemical-resistant gloves. Crews were also provided with a treatment plan, contact information for emergency professionals in the local areas, personnel information, and a map with detailed instructions for the application methods. Additionally, each boat was supplied with a first aid kit, two one-liter water bottles for emergency eye wash, and a radio. The leadership team also noted that EarthTec QZ® is highly corrosive to metals which was an additional concern because the application boats were made of aluminum. Thus, in the event of a spill on the boat, the crews were instructed to quickly rinse the spilled area with lake water to prevent corrosion.

EarthTec QZ® was applied over three separate treatments; February 28th, March 15th, and March 30th, 2023. The first and second treatment included four application boats and used two application boats respectively. Additionally, application crews were dispersed throughout the parameters of the lake to use backpack sprayers to apply EarthTec QZ® to any exposed structures such has fish habitat, buoys, floating wave-breakers, docks, and any stagnant pools of water.

1.2.3 *Application Methods*

Each boat was fit with a 378.5 L plastic trough to hold the chemical, a small Honda EU-2000 generator, and a submersible pump attached to a PVC manifold to dispense the chemical (Figure 1.3). The troughs were filled with 132.5 L of lake water prior to the application, and 132.5 L of EarthTec QZ®. The chemical was diluted to reduce the likelihood of injury to crews and the severity of corrosion to the boat if a spill would occur. Once the troughs were filled with the 50/50 mixture, large stir sticks were used to ensure proper mixing. Submersible pumps were allotted to dispense 265 L in approximately 15 minutes during application to allow the crew time

to monitor the volume and spatial application across each treatment zone. After each treatment, the boats returned to the boat ramp to refill the trough using the methods described above. Each boat completed five application passes throughout a treatment zone for the first and second treatment, and three passes for the third treatment.

1.2.4 *Treatment Zones – First and second application*

Boats were directed to dispense EarthTec QZ® traveling at 2.32 km h^{-1} per pass and at intervals of 0-10 meters from shore (pass 1), 10-20 meters from shore (pass 2), 20-30 meters from shore (pass 3), 30-40 meters from shore (pass 4), and 40-50 meters from shore (pass 5). Four application boats and a raft were used to apply 700 gallons of EarthTec QZ® throughout the outer margins of the lake. The product is known to disperse throughout the water column, so concerns that it would not reach the middle of the lake (untreated area) was not a concern. Application boat #1 applied EarthTec QZ® in treatment zone 1 (Figure 1.3) along the dam between the southwest corner and southeast corner of the dam. Application boat #2 applied the product in treatment zone 2, along the northern shoreline. Shallow mudflats and northern coves were avoid and treated from a raft. Two passes with 30 gallons of product for two rafts were completed to treat the coves and mudflats on the northern end of the reservoir. Application boat #3 applied the product in treatment zone 3 (Figure 1.3) along the western shoreline between the west side of the reservoir and southwest corner of the dam. Finally, boat #4 applied the product along the eastern shoreline between the southeast corner of the dam to the east side of the reservoir located just north of the inlet channel.

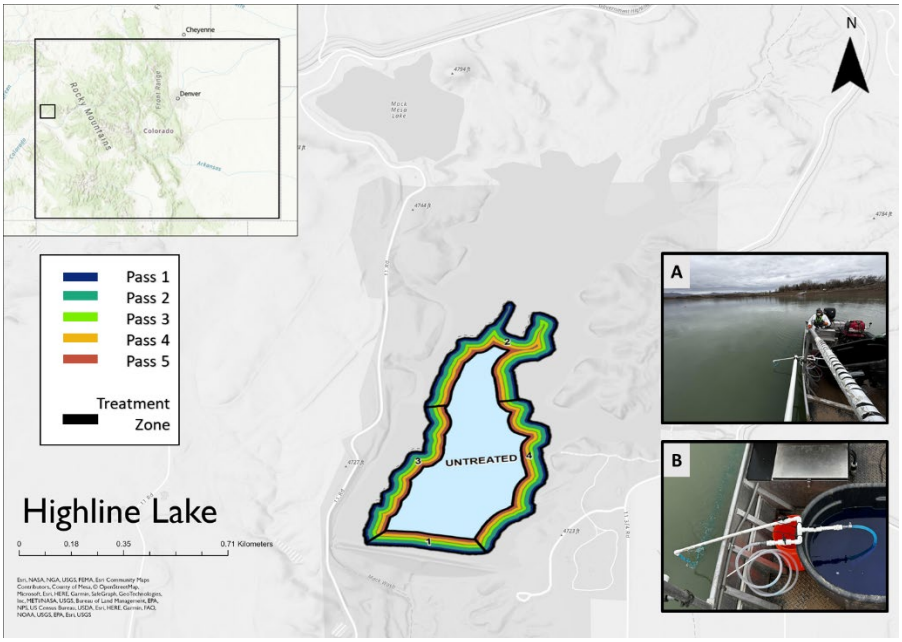


Figure 1.3 Treatment zones (black cross section lines) and pass lines for each of the four application boats for the first and second applications. Pass 1 applied the copper at 0-10 meters from shore, pass 2 applied 10-20 meters from shore, pass 3 was 20-30 meters from shore, pass 4 was 30-40 meters from shore and pass 5 was 40-50 meters from shore. Copper was administered by boat (A) at a max speed of 2.32 km h^{-1} and applied with a submersible pump attached to a PVC manifold (B).

1.2.5 *Treatment Zones – Third application*

EarthTec QZ® was applied to approximately 50% of Highline Lake along the outer margins. Two application boats were used and applied 200 gallons of product to ensure the target copper concentration was maintained until the canal water started filling the lake. Each boat completed three passes along the perimeter of the lake, each 0-10 meters from shore. Thirty-five gallons of product diluted with 35 gallons of water was applied from two separate stock tanks on each boat. An additional 30 gallons of product was then diluted with 35 gallons of lake water to apply with a third pass along the dam (Figure 1.4). Application boat #1 applied EarthTec QZ® from the north end of the reservoir moving counter-clockwise to the center of the dam over three passes

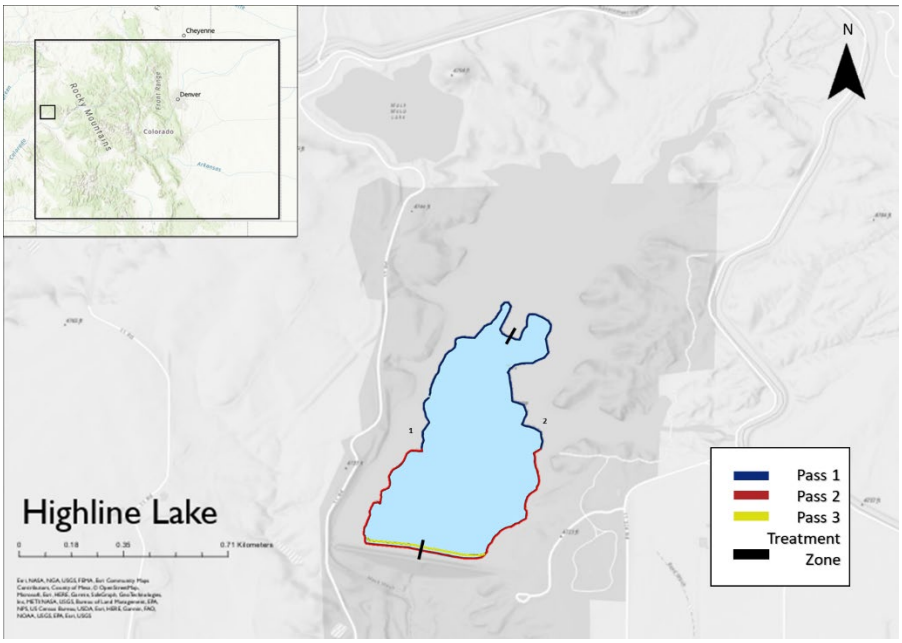


Figure 1.4 Treatment zones (black cross section lines) and pass lines for each of the two application boats for the first and second applications. Each pass was 0-10 meters from shore and pass 3 was an additional pass along the dam.

1.2.6 *Water quality sampling*

Throughout the lake, nine sampling points were determined based on location, depth, and access from a boat or the shoreline (Figure 1.5). Two sampling locations were strategically selected to ensure two sampling points at each depth across the lake. At each sampling location a Van Dorn sampler was used to collect water from the top and bottom layers of the lake. At each sampling location, the Van Dorn sampler was rinsed and a site specific pre-labeled 1 L Nalgene label was rinsed three times prior to collecting the sample. A water quality sonde was placed at the top and bottom locations of all sampling sites to collect temperature, pH, and dissolved oxygen (DO). We collected water surface samples by submerging the Van Dorn sampler such that the entire sampler was under water and the messenger to close the sampler was dropped. Water was added

to the pre-labeled 1 L Nalgene specific to the site. Samples from the deepest part of the sampling location were collected 1 meter from the bottom with the same methods described above.

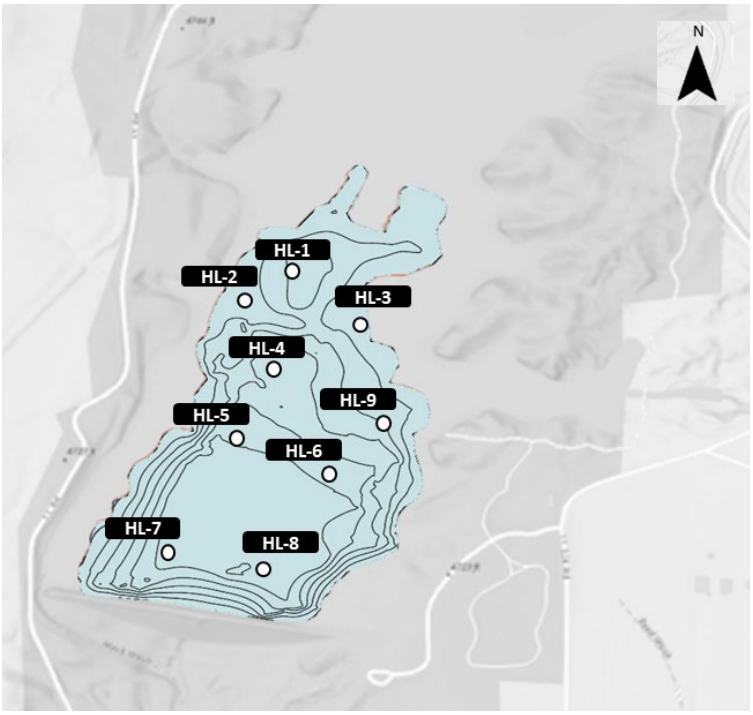


Figure 1.5 Nine sampling locations across Highline Lake (HL) for water quality sampling at various depths. Sentinel cages containing five Asian Clams in each cage were also placed at each sampling location.

After collection, samples were brought into the laboratory for further processing. Non-filtered water samples were collected by rinsing a 10 mL syringe twice with sample water, filled again with sample water, and were gently squirted into two 15 mL falcon tubes. Filtered water samples were collected from the same sample water in the syringe used for the non-filtered sample. A 47 mm, 0.45 µm polyethersufone (PES) filter was screwed onto the bottom of the syringe and seeded with a few drops of the sample into a waste bucket. The remaining sample was filtered and placed into two, 15 mL falcon tubes. Samples were preserved with 1 mL L^{-1} ultra-pure nitric acid (Ultrex® II, J.T. Baker or equivalent) to reach a pH less than 2 and shipped to the River Watch and CPW Toxicology Laboratory (Fort Collins, CO).

1.2.7 *Metal Analysis*

Metal concentrations were analyzed using a ThermoScientific iCAP 6000 Series Inductively Coupled Plasma, Optical Emission Spectrometer (ICP-OES) introduced using a CETAC ultrasonic nebulizer (U5000AT+) and operated with iTeva software (version 2.8.0.97). Temperature set points used in the nebulizer were 3°C and 140°C. Samples were injected into the plasma using a quartz torch and a 2.0 mm quartz center tube. Prior to analysis, nitrogen gas (N_2) was used to purge the instrument for a minimum of five hours before igniting the high purity argon (Ar) plasma. The plasma was used to stabilize for one hour before initial calibration. The instrument was calibrated using a five-point curve to bracket the concentrations of the

analytes (Table 4.1). Matrix solution for blanks and for standard dilutions were made in 1% HNO_3 and 1% HCl using deionized water by volume (DI; Barnstead Nanopure system). Calibration standards were composed of separate externally purchased NIST-Traceable certified standards (SRM 3100 Series). We used yttrium (Y) as an internal standard and Y was introduced inline and monitored at three wavelengths (Table 4.1). Method detection and reporting limits were previously determined following EPA procedures (Table 1.1).

Table 1.1 Calibration standards for ICP-OES method to detect aluminum (Al), arsenic (As), calcium (Ca), cadmium (Cd), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), lead (Pb), selenium (Se), and zinc (Zn). All units are represented in $\mu g\ L^{-1}$. Method detection limits (MDL), reporting limits (RL), and axial or radial detection are included for each element.

Analyte	Wavelength	Blank	STD1	STD2	STD3	STD4	STD5	STD6	MDL	RL	Y	Axial/Radial
Al	396.15	0	50	500	1,000	2,500	5,000	50,000	9.90	40.6	371.03	Radial
As	189.04	0	15	150	300	750	1,500	-	0.85	3.50	224.31	Axial
Ca	315.89	0	2,000	20,000	40,000	100,000	200,000	500,000	38.1	156	360.07	Radial
Cd	214.44	0	5	50	100	250	500	-	0.25	1.02	244.31	Axial
Cu	327.40	0	5	50	100	250	500	-	1.35	5.54	360.07	Axial
Fe	238.20	0	50	500	1,000	2,500	5,000	-	6.13	25.1	360.07	Axial
Fe	274.93	0	50	500	1,000	2,500	5,000	20,000	6.13	25.1	360.07	Axial
K	766.49	0	500	5,000	10,000	25,000	50,000	-	15.1	62.1	371.03	Radial
Mg	279.08	0	-	10,000	-	50,000	100,000	300,000	14.5	59.4	360.07	Radial
Mg	285.21	0	1,000	10,000	20,000	50,000	100,000	-	14.5	59.4	360.07	Radial
Mn	257.61	0	50	500	1,000	2,500	5,000	-	0.60	2.46	360.07	Axial
Na	589.59	0	500	5,000	10,000	25,000	50,000	400,000	18.3	74.9	371.03	Radial
Pb	220.35	0	5	50	100	250	500	-	1.30	5.31	224.31	Axial
Se	196.09	0	-	10	20	50	100	-	1.88	7.71	244.31	Axial
Zn	213.86	0	50	500	1,000	2,500	5,000	-	1.41	5.79	244.31	Axial

After calibration, a series of initial quality control (QC) samples were analyzed including an internal calibration verification (ICV) using NIST certified externally purchased standard, a mid-range standard, a continuing calibration verification (CCV), and continuing calibration blank (CCB). Analysis proceeded if each element was calibrated with a correlation coefficient value of 0.995 or greater and all subsequent QC samples were within 10% of the expected value.

Overall, the analysis lasted 220 seconds followed by an 80 second rinse using 1% HNO_3, 1% HCl in Nanopure water by volume with three repetitions of each sample before rinsing. Duplicate samples were ran every ten samples, alternating between sample duplicates and spiked duplicates. The acceptable range for duplicate samples was $\pm$ 10%. In each analytical batch of samples, the first spiked duplicate consisted of an external spike and subsequent duplicate spike samples consisted of an internal standard. Spike recovery was acceptable between 85-115%. If any of the analytes fell outside of the calibration range, the sample was diluted using 1% HNO_3, 1% HCl solution. Blanks were flagged if they were greater than 5% of the detection limits as suggested by the manufacturer or detection limit calculated from previous sample batches. Any sample that did not pass QC tolerances, or was not bracketed by passing QC CCV/CCB samples were reanalyzed.

1.2.8 *Sentinel Cages*

One sentinel cage was placed at each sampling location throughout Highline Lake (Figure 1.5). Cages were fabricated from 4x2 inch black, PVC pipes with capped ends. Small holes were made on the sides of the PVC to allow for water flow. At one end of the cage the pipe was attached to a buoy and at the other end was attached to a weight to keep the cage submerged. Asian Clams (*Corbicula* sp.) were collected prior to the treatments from Highline Lake and five clams were placed in each of the sentinel cages. Two control sentinel cages were placed below the dam in Mack Wash. Cages were checked every day in the early afternoon for any mortalities.

1.2.9 *Modeling*

A model was developed to interpolate copper concentrations throughout Highline Lake after the three copper sulfate treatments. This model applies geostatistical methodologies that use spatial coordinates to predict the copper concentrations which weights the surrounding measured values to derive a predicted value for the copper concentration at unmeasured locations. Specifically, average copper concentration values from the ICP-OES results at the sampling locations for all three treatments were used to create a Kernel Density Model with the ArcGIS Geospatial tool to estimate copper concentrations across the lake as a function of the average weekly water temperature, pH, depth, and water hardness (Figure 1.6).

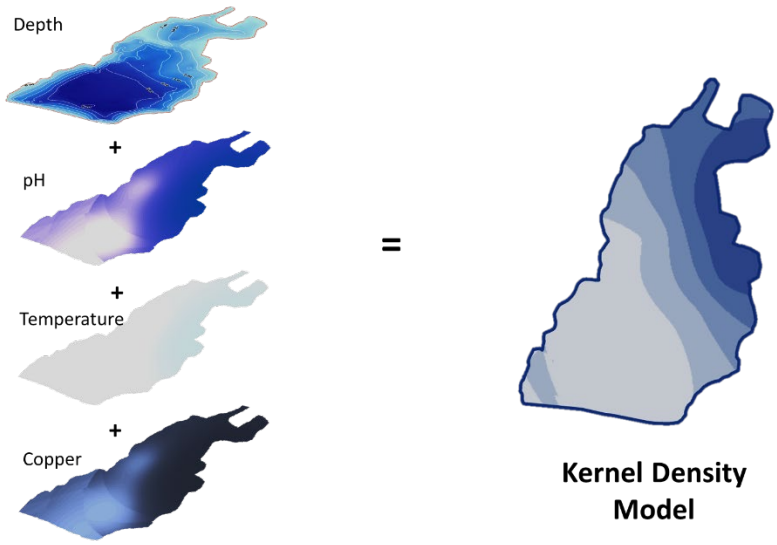


Figure 1.6 Our geostatistical methodologies used spatial coordinates to predict the copper concentrations which weights the surrounding measured values to derive a predicted value for the copper concentration at unmeasured locations. Copper concentration values from the ICP-OES results at the sampling locations were used to create a Kernel Density Model with the ArcGIS Geospatial Tool to estimate copper concentrations across the lake as a function of the depth, pH, and average weekly temperature.

1.3 Results

1.3.1 *Treatment Overview*

The attempted eradication of Zebra Mussels from Highline Lake via the application of EarthTec QZ® was conducted over three treatments: February 28, March 15, and March 30, 2023. The lake was drained to approximately 25% capacity before the first treatment, and the application method involved a dilution of copper sulfate at a concentration of 4 ppm. Throughout the treatment, careful monitoring of the water's physiochemical parameters was conducted, focusing on copper concentrations, temperature, pH, and dissolved oxygen (DO). All treatments were successful using the application methods described above.

1.3.2 *Water Quality Monitoring*

Water temperature was recorded every morning during the three treatments and average temperatures ranged between 4.2°C to 8.7°C and daily averages ranged from 4.0°C to 10.0°C The EarthTec QZ® label suggests applying the agent when the water temperature remains above 7°C for at least 14 consecutive days. During the first treatment there were only two days above 7°C, three days in the second treatment, and ten days during the third treatment (Figure 1.7).

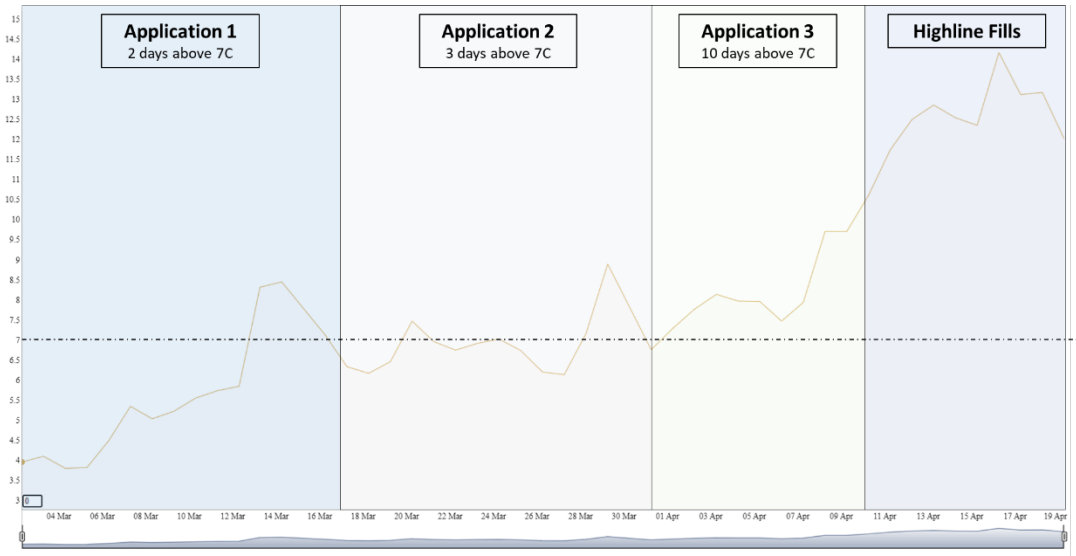


Figure 1.7 Temperatures at Highline Lake collected from all sites. Dashed horizontal line indicates 7°C which is lowest temperature recommendation for successful eradication efforts, per the label.

Post-treatment water quality analysis indicated fluctuations in copper concentrations across the sampling sites within Highline Lake. Copper concentrations in the first treatment ranged from 127.3 µg L^{-1} to 164.6 µg L^{-1} at various depths across the sampling sites (Table 1.2).

Table 1.2 Temperature (°C), pH, dissolved oxygen (mg L^{-1}), depth (m), and copper concentrations (µg L^{-1}) at sampling locations for every site in the first, second, and third treatment.

Site	Location	Depth (m)	Temperature (°C)	pH	DO (mg/L)	Copper (µg/L)
First Treatment						
HL-1	Bottom	2	4.2 ± 0.8	8.3 ± 0.1	10.4 ± 0.1	153.4 ± 33.0
HL-1	Surface	2	4.3 ± 0.8	8.3 ± 0.1	10.5 ± 0.2	159.9 ± 25.9
HL-2	Surface	1	4.6 ± 0.9	8.3 ± 0.1	10.4 ± 0.2	154.6 ± 23.4
HL-3	Surface	0.75	4.6 ± 0.9	8.3 ± 0.2	10.5 ± 0.3	164.6 ± 67.3
HL-4	Bottom	3	4.4 ± 0.8	8.3 ± 0.1	10.4 ± 0.1	150.1 ± 20.6
HL-4	Surface	3	4.7 ± 0.8	8.3 ± 0.1	10.5 ± 0.2	144.6 ± 15.5
HL-5	Bottom	4.5	4.4 ± 0.6	8.3 ± 0.2	10.3 ± 0.3	148.0 ± 18.1
HL-5	Surface	4.5	4.7 ± 0.8	8.3 ± 0.1	10.4 ± 0.1	145.4 ± 18.5
HL-6	Bottom	4.5	4.3 ± 0.6	8.3 ± 0.1	10.4 ± 0.2	148.6 ± 17.7
HL-6	Surface	4.5	4.4 ± 0.8	8.3 ± 0.1	10.6 ± 0.2	148.1 ± 16.7
HL-7	Bottom	5.5	4.3 ± 0.6	8.1 ± 0.2	10.2 ± 0.2	146.3 ± 15.7
HL-7	Surface	5.5	4.3 ± 0.9	8.2 ± 0.2	10.5 ± 0.2	147.4 ± 14.4
HL-8	Bottom	6	4.2 ± 0.7	8.3 ± 0.1	10.5 ± 0.2	127.3 ± 25.0
HL-8	Surface	6	4.2 ± 0.9	8.3 ± 0.2	10.6 ± 0.2	139.3 ± 16.6
HL-9	Surface	1	4.5 ± 0.8	8.1 ±0.5	10.5 ± 0.2	153.5 ± 43.4
Second Treatment						
HL-1	Bottom	2	6.3 ± 0.3	8.0 ± 0.04	9.8 ± 0.1	187.0 ± 34.8
HL-1	Surface	2	6.3 ± 03	8.0 ± 0.04	9.9 ± 0.1	179.1 ± 31.9
HL-2	Surface	1	6.2 ± 0.2	8.0 ± 0.03	9.9 ± 0.1	188.0 ± 33.9
HL-3	Surface	0.75	6.5 ± 0.5	8.0 ± 0.03	9.9 ± 0.03	193.9 ± 26.9
HL-4	Bottom	3	6.3 ± 0.4	8.0 ± 0.03	9.9 ± 0.1	191.8 ± 28.8
HL-4	Surface	3	6.4 ± 0.4	8.0 ± 0.03	9.9 ± 0.1	191.2 ± 30.6
HL-5	Bottom	4.5	6.4 ± 0.3	8.0 ± 0.03	9.9 ± 0.1	190.2 ± 29.9
HL-5	Surface	4.5	6.6 ± 0.3	8.0 ± 0.03	9.8 ± 0.1	196.0 ± 31.3
HL-6	Bottom	4.5	6.5 ± 0.3	8.0 ± 0.04	9.9 ± 0.1	188.6 ± 31.0
HL-6	Surface	4.5	6.6 ± 0.3	8.0 ± 0.03	9.9 ± 0.1	197.1 ± 30.2
HL-7	Bottom	5.5	6.5 ± 0.3	8.0 ± 0.04	9.8 ± 0.01	192.9 ± 26.8
HL-7	Surface	5.5	6.8 ± 0.3	8.0 ± 0.03	9.9 ± 0.1	194.5 ± 27.2
HL-8	Bottom	6	6.5 ± 0.3	8.0 ± 0.03	9.9 ± 0.1	197.4 ± 31.2
HL-8	Surface	6	6.7 ± 0.3	8.0 ± 0.03	9.9 ± 0.1	193.9 ± 29.2
HL-9	Surface	1	6.3 ± 0.3	8.0 ± 0.03	9.9 ± 0.1	183.2 ± 37.4
Third Treatment						
HL-1	Surface	2	8.0 ± 1.3	8.0 ± 0.04	9.8 ± 0.1	110.6 ± 42.4
HL-1	Bottom	2	7.9 ± 1.2	8.0 ± 0.04	9.7 ± 0.1	114.5 ± 64.7
HL-2	Surface	1	7.9 ± 1.2	8.0 ± 0.04	9.8 ± 0.1	108.5 ± 46.1
HL-3	Surface	0.75	7.9 ± 1.7	8.0 ± 0.1	9.8 ± 0.1	109.4 ± 42.2
HL-4	Surface	3	8.1 ± 1.2	8.1 ± 0.1	9.8 ± 0.1	113.0 ± 39.3
HL-4	Bottom	3	8.2 ± 1.4	8.0 ± 0.04	9.7 ± 0.1	112.4 ± 37.3
HL-5	Surface	4.5	8.6 ± 1.5	8.1 ± 0.1	9.8 ± 0.1	105.0 ± 15.7
HL-5	Bottom	4.5	7.6 ± 0.6	8.0 ± 0.1	9.7 ± 0.2	103.6 ± 23.8
HL-6	Surface	4.5	8.5 ± 1.6	8.1 ± 0.1	9.8 ± 0.1	123.7 ± 40.8
HL-6	Bottom	4.5	7.4 ± 0.6	8.0 ± 0.4	9.7 ± 0.1	121.6 ± 41.5
HL-7	Surface	5.5	8.7 ± 1.6	8.1 ± 0.2	9.8 ± 0.1	105.2 ± 24.4
HL-7	Bottom	5.5	7.6 ± 0.6	8.0 ± 0.03	9.7 ± 0.7	101.6 ± 12.5
HL-8	Surface	6	8.6 ± 1.6	8.1 ± 0.2	9.8 ± 0.1	104.9 ± 26.2
HL-8	Bottom	6	7.3 ± 0.4	8.0 ± 0.04	9.6 ± 0.2	110.0 ± 25.2
HL-9	Surface	1	8.3 ± 1.7	8.0 ± 0.04	9.7 ± 0.1	95.2 ± 38.7

The highest concentrations were typically observed in the shallow areas. Concentrations in the second treatment showed an increase, with surface measurements reaching up to 193.9 µg L^{-1}. Sites with depths 3 meters or lower indicated elevated copper levels, coinciding with favorable temperatures and pH levels maintained at approximately 8.0 throughout the treatment. Following the last treatment, copper concentrations decreased, with values at 95.2 µg L^{-1} to 114.5 µg L^{-1}. Water temperatures were the highest during the third treatment as expected and DO decreased while pH remained the same (Figure 1.8).

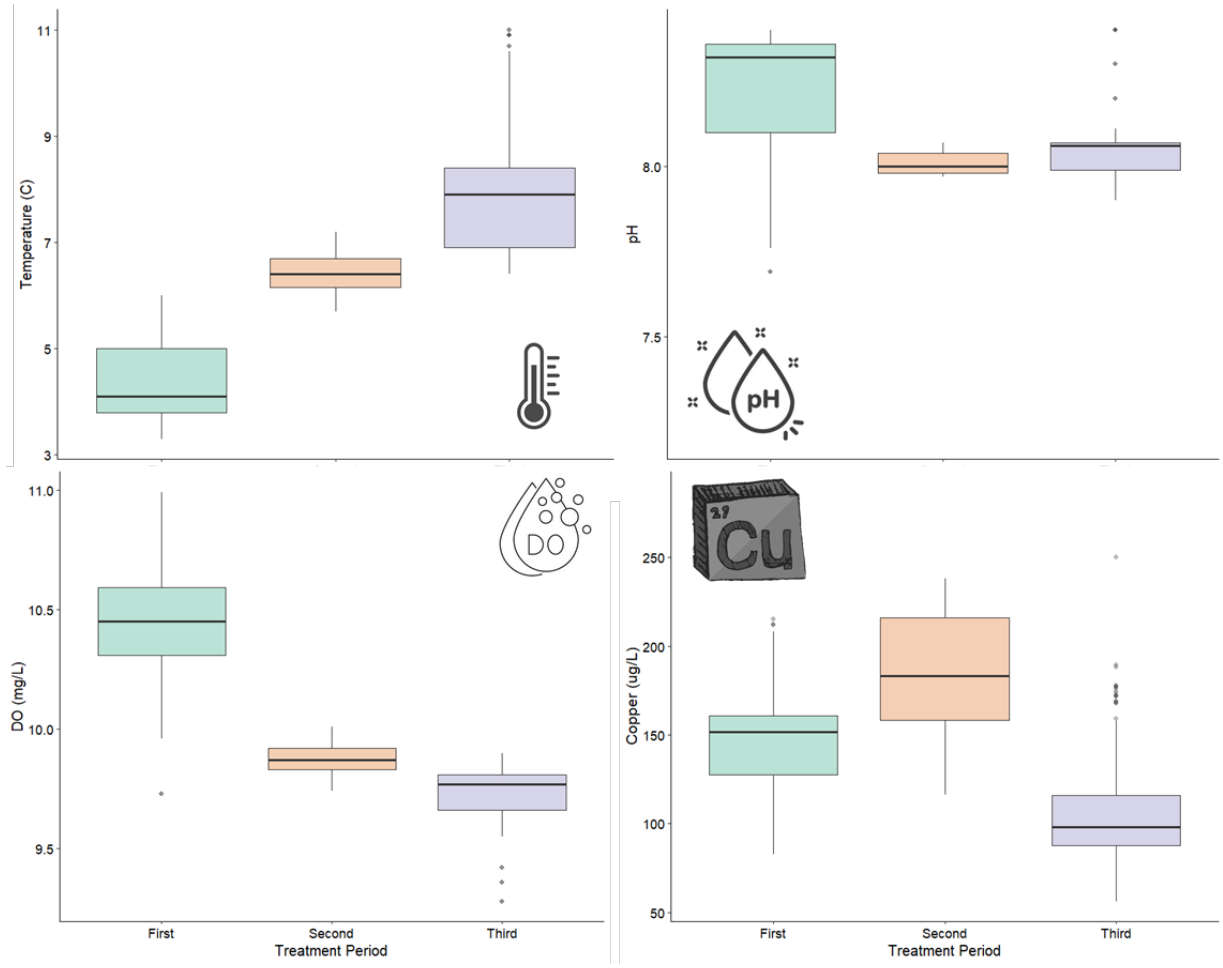


Figure 1.8 Boxplots of water temperature (°C; top left), pH (top right), dissolved oxygen (mg L^{-1}; bottom left), and copper concentrations (µg L^{-1}; bottom right) for each of the three treatment periods. Boxplot extent ranges represent the 25th and 75th percentile; band near the middle of the box represents the 50th percentile/median; whiskers range from lowest the highest datum with points as outliers.

1.3.3 *Sentinel cages*

Sentinel cages of Asian Clams showed some signs of mortality. However, the cage design for the clams failed at five out of nine of the sites. The cages broke off from buoys or weights holding the cages underwater causing them to fail. Thus, the percentage of mortality due to cage

malfunction was 72 ± 22.8%. Percent mortality of clams observed in cages that did not malfunction was 65 ± 41.1%. No mortalities of clams were observed in the control sentinel cage below the dam in Mack Wash.

1.3.4 *Kernel Density Model*

Our geostatistical model indicates that average levels of copper remained relatively high across the reservoir at non-sampled locations (Figure 1.9). We aimed to maintain a target copper concentration of 200 µg L^{-1}. Higher concentrations were observed in the shallows especially in the muddy flats in the north eastern corners. Copper levels were lower in the deep locations near the southwest corners by the dam.

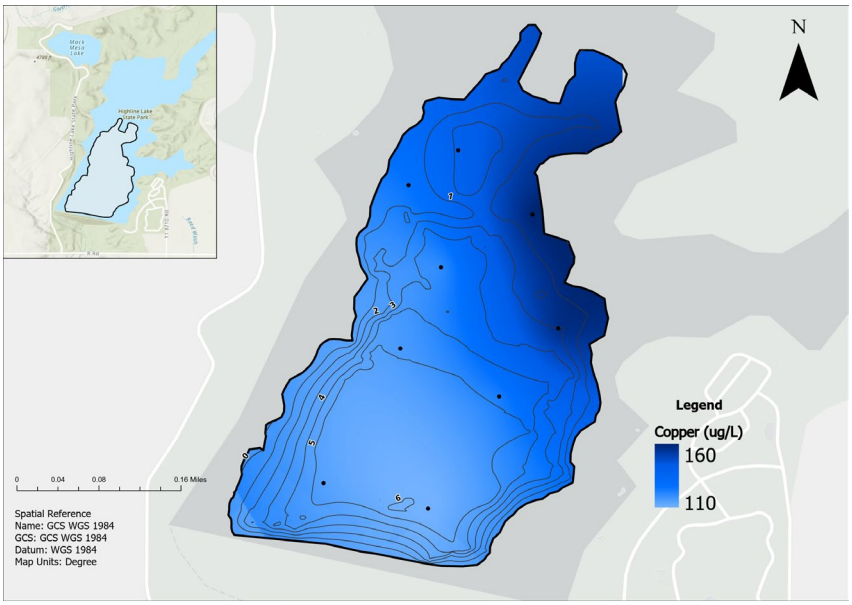


Figure 1.9 Results from our Kernel Density Model. Average copper concentrations from all three treatments across Highline Lake ranged from 110-160 µg L^{-1}.

1.4 Discussion

Our ongoing efforts for invasive species management in Colorado revealed the presence of adult Zebra Mussels in late fall 2022 in Highline Lake. The unique hydrological conditions at Highline Lake, which includes a government canal that is shut off from late fall through early spring, presented a potential opportunity for targeted eradication efforts. This stagnant water period allowed us to hypothesize that we could effectively apply treatments without the complications of inflow or outflow, challenges faced in larger or continuously flowing water bodies. Since detections were in the late fall, applications began in the winter season when we had to deal with colder water temperatures. Unfortunately, a few months after our treatments, the presence of Zebra Mussels were detected in Highline Lake. We predict that water temperature and time of year of application may have affected the efficacy of the copper sulfate treatments.

In late winter and early spring, we applied our first round of treatments using EarthTec QZ® to Highline Lake at a dose of 4 ppm of copper sulfate. However, we dealt with colder water temperatures during this period, which can significantly impact the efficacy of copper against Zebra Mussels by slowing down their respiration and metabolic rate and decrease the overall of toxicity of copper to the mussels (Rao and Kahn 2000). According to the product specifications, effective treatment requires temperatures above 7°C for at least 14 consecutive days. During our monitoring we noted that during treatment one, temperatures were above 7°C for only two days, treatment two managed three days, and treatment three experienced ten days of suitable temperatures before dilution began as the reservoir refilled. Temperatures were collected in the early afternoon every day, so the lowest temperatures for a 24 hours period may have been missed suggesting that consecutive days above 7°C may be even lower than we assume.

We conducted thorough bathymetric mapping to estimate water volume for accurate dosage calculations. While we observed missing of copper sulfate throughout the reservoir, deeper areas held lower concentrations, and there was no evidence of thermal stratification, which could have otherwise limited copper dispersion. Copper concentrations ranged from 95.2 to 197.4 mg L^{-1} and averaged 139.0 mg L^{-1}. Targeted concentrations were 200 mg L^{-1}. Decreased concentrations were likely due to binding of the copper to other substrates in the lake. When cupric ions of copper are introduced into a lake environment, copper may bind to dissolved natural organic matter and to clay, organic matter, sulfides, and hydrous manganese oxides in sediment (Rader et al. 2019). Nonetheless, levels remained relatively high and should have been able to eradicate the mussels or veligers if present. Our sentinel cages indicated that at least 65% of the Asian Clams resulted in mortality over the course of the three treatments. However, the cage design and placement of the cages may have influenced the overall mortality of the clams because of mishandling and no substrate for the clams to bind to inside of the cages. Thus, mortality of the clams in the sentinel cages may not be an accurate representation of copper toxicity to the Zebra Mussels in the lake.

Based on our findings, we can state the application methods we used were successful at ensuring proper missing of copper sulfate throughout the lake to maintain levels high enough to eradicate the mussels. However, in late summer and early fall of 2024 the presence of Zebra Mussels were observed once again in Highline Lake. We predict that the water temperatures which lead to decreased copper toxicity and lower respiration and metabolic rates of the mussels affected the success of our eradication efforts. Prior to any future treatments, we recommend conducting bioassays prior to treatment to assess the potential impact of copper sulfate on Zebra Mussels more accurately, as well as utilizing appropriate sentinel cages to monitor real-time mussel responses to the treatments. Our work emphasizes the importance of considering temperature and water dynamics when planning future eradication strategies for invasive species like Zebra Mussels.

1.5 References

Carmosini N., R. Gillis, A. Ismail, and G. J. Sandland. 2018. A pilot evaluation of the toxicity of EarthTec QZ on invasive (*Bithynia tentaculata*) and native (*Physa gyrina*) snail species. Bulletin of Environmental Contamination and Toxicology 101(4): 428-433.

Casagrandi R., L. Mari, and M. Gatto. 2007. Modelling the local dynamics of the Zebra Mussel (*Dreissena polymorpha*). Freshwater Biology 52: 1223-1238.

Frischer M. E., B. R. McGarth, A. S. Hansen, P. A. Vescia, J. A. Wyllie, J. Wimbush, and S. A. Nierzwicki-Bauer. 2009. Introduction pathways, differential survival of adult and larval Zebra Mussels (*Dreissena polymorpha*), and possible management strategies, in an Adirondack Lake, Lake George, NY. Lake and Reservoir Management 36:4 432-443.

Griffiths, R. W., D. W. Scholesser, J. H. Leach, and W. P. Kovalak. 1991. Distribution and dispersal of the Zebra Mussel (*Dreissena polymorpha*) in the Great Lakes region. Canadian Journal of Fisheries and Aquatic Sciences 48: 1381-1388.

Hammond D. and G. Ferris. 2019. Low doses of EarthTec QZ ionic copper used in effort to eradicate quagga mussels from an entire Pennsylvania lake. Management of Biological Invasions 10: 500-516.

Herbert, P. D. N., B. W. Muncaster, and G. L. Mackie. 1989. Ecological and genetic studies on *Dreissena polymorpha* (Pallas): A new mollusc in the Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences 46: 1587-1591.

Iwanyckyj, E., M. Albright, and D. Stich. 2017. Effectiveness of molluscicide EarthTec QZ on adult and veliger Zebra Mussel (*Dreissena polymorpha*) mortality. SUNY Oneonta Biological Field Station, SUNY Oneonta, 14 pp

Kovalak W. P. 1989. Life history and biology of the Zebra Mussel (*Dreissena polymorpha*). Engineering Research Report 89A90-3. 7p. NY Sea Grant Zebra Mussel Clearing House, SUNY College, Brockport, NY.

Ludyanskiy M. L., D. McDonald, and D. MacNeill. 1993. Impact of Zebra Mussel, a bivalve invader. BioScience 43(8): 533-544.

Morton B. 1971. Studies on the biology of *Dreissena polymorpha* Pall. V. Some aspects of filter feeding and the effect of micro-organisms upon the rate of filtration. Proceedings of the Malacological Society of London 39: 289-301.

O'Neill C. R. and D. B. MacNeill. 1989. *Dreissena polymorpha* an unwelcome new great lakes invader. Costal Resources Fact Sheet. New York Sea Grant Extension Program, Ithaca, NY, USA.

Rader K. J., R. F. Carbonaro, E. D. van Hullebusch, S. Baken, and K. Delbeke. 2019. The fate of copper added to surface water: Field, laboratory, and modeling studies. Environmental Toxicology and Chemistry 38(7): 1386-1399.

Rao P. D. G. V. and Khan M. A. Q. Zebra Mussels: Enhancement of copper toxicity by high temperature and its relationship with respiration and metabolism. Water Environment Research 72(2): 175-178.

Smirnova N. F. and G. A. Vinogradov. 1990. Biology and ecology of *Dreissena polymorpha* from the European USSR. Presented at the workshop on Introduced Species in the Great Lakes: Ecology and Management. Saginaw, MI. 26-28 Sept.

Sprung M. 1995. Physiological energetics of the Zebra Mussel *Dreissena polymorpha* in lakes III. Metabolism and net growth efficiency. Hydrobiologia 304: 147-158.

Spring M. and U. Rose. 1988. Influence of food size and food quantity of the feeding of the mussel *Dreissena polymorpha*. Oecologia 77: 526-632.

Stokstad E. 2007. Feared quagga mussel turns up in western United States. Science 35:241-249

Ten W. M. E. H. and Davids C. 1982. Food selection by *Dreissena polymorpha* Palla (Mollusca: Bivalvia). Freshwater Biology 12: 553-558.

Watters A., S. L. Gerstenberger, and W. H. Wong. 2013. Effectiveness of EarthTec for killing invasive Quagga Musels (*Dreissena rostriformis bugensis*) and preventing their colonization in the Western United States. Biofouling 29: 21-28.

Wimbush J., M. E. Frischer, J. W. Zarzynski, and S. A. Nierzwicki-Bauer. 2009. Eradication of colonizing populations of Zebra Mussels (*Dreissena* polymorpha) by early detection and SCUBA removal: Lake George, NY. Aquatic Conservation: Marine and Freshwater Ecosystems 19: 703-713.

Aquatic Macroinvertebrate Drift Behavior after Acute Exposure to Aqueous Copper

Dana Musto, Kacey Tepley, Dr. Pete Cadmus

2.1 Introduction

USEPA guidelines for derivation of acute standards (Stephan et al.1985) consider experiments of 96 hr (48 hr for short lived species) in duration. Water quality standards are intended to prevent the loss of biodiversity from surface water. Acute experiments consider only endpoints from which no organism would recover. In the limited ecological relevance of the laboratory setting this has historically limited studies to the endpoint of only mortality. Chemical avoidance can abruptly extirpate species from a reach. Both fish and aquatic insects "drift" with the current as a response to stressful water chemistry. This can occur abruptly at sub-acute (< 96 hr) durations and at sub-lethal concentrations (below the level that would induce mortality). In Colorado, fish passage, especially against current, is routinely obstructed by diversion structures, culverts and steep slopes. In the fast flowing mountain streams common in Colorado, up-stream movement of aquatic insects is not possible even in unaltered (natural) drainages. To meet the requirements of the Clean Water Act (Duggan and Kotalik 2020) and to approach the quality of standard building methodologies adopted by most every other developed country, the US Environmental Protection Agency (USEPA) will need to modernize the types of experiments and data used in the creation of water quality standards (Elias et al. 2015) and actively initiated this effort a decade ago.

Drift and chemical avoidance of aquatic organisms is regularly observed, often well below lethal levels of pollution (Sprague 1971; Svecevičius 1999; Clements 2004). A large discrepancy exists between the toxic thresholds found in 4d or 30d laboratory toxicity trials and the rather low levels of pollution observed where Colorado's fish and aquatic invertebrates have been extirpated or face poor recruitment. This has been especially true for salinity/road salt (chloride), nitrates, phosphates, and sulfate. Preliminary studies by CPW find potential extirpation events near these pollution sources but traditional toxicity trials fail to illicit mortality. Chemical avoidance was observed for similar ions; HCO_3^-, Cl^-, SO_4^{2-}, Mg^{2+}, Na^+, and Ca^{2+} (Clements and Kotalik 2016). Copper is known to illicit a drift response in the first few days of 30d and 10d mesocosm studies (Clements et al. 2013; Clements 2004). However, the results of such studies were precluded from the standard building process as the duration was not 96 hr. We present an experiment that used naturally colonized assemblages of aquatic macroinvertebrates exposed to Cu. Drift was assessed continuously throughout the experiment to be used as a measure of chemical avoidance that can extirpate taxa from watersheds. Drifting organisms were removed from the experiment to better simulate nature. Survival was assessed at 96 hr. This methodology will be expanded to characterize species loss for other analytes that may illicit a drift behavior at sub-lethal concentrations.

2.2 Methods

2.2.1 *Mesocosm Toxicity Experiment*

To quantify the relative importance of drift, versus survival, in acute toxicity trials benthic macroinvertebrate assemblages were exposed to a gradient of copper ion (0, 2.24, 4.45, 8.85, 17.6, and 35 µg/L) for four days. Because of the limited number of experimental streams (16) an unbalanced replication was employed; n=4, 3, 3, 3, 2, and 1 respectively. This improved ability to characterize effect concentrations (*i.e.* LC_{50}) at the cost of ability to detect differences between treatment levels in an ANOVA framework. Experiments were conducted using naturally colonized substrate and exposures were conducted in circular artificial streams that maintain a lotic flow similar to a medium gradient mountain stream. Drift was continually captured in fine mesh nets. Details of experimental streams and methods have been described previously (Clements et al. 2013). Briefly, 10 x 10 x 10-cm plastic trays filled with natural pebble and cobble substrate were placed at a reference site (Michigan River, Gould, Colorado, USA) for 30 d. Three holes (2.5 cm diameter) drilled in each side of the trays increased water flow and facilitated macroinvertebrate colonization. Trays were colonized by a diverse assemblage of aquatic insects that are representative of communities in the natural substrate. After colonization, trays were removed from the stream, placed in 4-L insulated containers filled with stream water (4 trays per container) and transferred to the experimental streams in at the Colorado Parks and Wildlife Aquatic Toxicology Laboratory. The contents of each container were randomly assigned to one of 16 stream mesocosms. The contents of three additional containers were preserved to represent the population at the start of the experiment (Day-0). Dechlorinated municipal tap water (Fort Collins, CO, USA; Hardness of 50-54 mg/L) fed each stream at 40 mL/min. Water quality characteristics (pH, conductivity, temperature, dissolved oxygen) were representative of Colorado mountain streams. Paddlewheels maintained a constant current velocity of 0.35 m/s in the experimental streams when no drift net was present. Timers produced summer diurnal cycles (16:8 hr) using wide spectrum LED grow lights (but not UV). Communities were given two night cycles (~48 hr) to acclimate to experiment streams. After which peristaltic pumps delivered copper stock solutions to a gravity fed serial diluter that delivered 40 mL/min to each 67.6 L stream. The “ramp-up” to target concentrations was accelerated for the first six hours by delivering target concentrations of Cu ion to each experimental stream at 740 mL/m. This brought experimental streams up to 95% of the target Cu concentration after three hr and 99% of the target concentration after 5.1 hr. This was achieved by supplementing the 40 mL/min flow from the gravity fed diluter using water with larger flows from a chilled and aerated head tank through metered valves. Drift nets were then installed and swapped every twelve hours (6 am and 6 pm) to characterize nighttime and daytime drift separately. Contents of nets were preserved in 80% ethanol, enumerated and identified to species (tribe for chironomids). After 96 hours of exposure the last drift net observation was preserved and toxicant pumps were stopped. Contents of each mesocosm were preserved, enumerated and identified.

2.2.2 *Water Chemistry*

Daily pH, temperature, conductivity, and dissolved oxygen was assessed using calibrated hand held probes. Hardness and alkalinity were titrated by USEPA methods 130.2 and 310.1. Dissolved (0.45 µm nylon filter) and total (unfiltered) copper samples were assessed daily from

each experimental unit at 6, 24, 48 and 72 hr (+/- four hr). A Thermo Fisher Scientific iCAP 6000 ICP-OES was used to analyze for Cu, Ca, Mg, K, and Na. Samples and solutions were preserved with ultra-pure (Ultrex®II, J.T. Baker or equivalent) nitric acid (1 mL per L or one drop per 5 mL of sample). Matrix solution for blanks standards and dilutions were made of deionized water (Barnstead Nanopure system; Thermo Fisher Scientific). Five-point calibration for each element was conducted prior to each batch of twenty samples and was analyzed after each batch to ensure no drift. At a minimum, the following quality assurance samples (QA) were analyzed. Each batch was accompanied with at least one duplicate sample at the time of collection and at least one sample split just prior to analysis, each flagged if duplicate or split was >5 or 10% from original. Blanks were flagged if greater than 5% of detection limit suggested by manufacturer or detection limit calculated from previous batches. External quality assurance standards for each element were assessed every 10 samples and were flagged if >5% from nominal or more frequently at the analyst's discretion. External standards obtained from nationally certified firms were NIST -Traceable to the SRM 3100 Series. Standards had a certificate of analysis and SDS that guaranteed accuracy (99.999% certified accuracy to ±0.3%) and stability. Yttrium internal calibration standard was continuously introduced into the plasma along with each sample. If any QA/QC flags were observed instrument was recalibrated and all samples of that batch were reanalyzed. Samples found above the highest standard in the calibration curve were diluted (1:2, 1:5 or 1:10) and reanalyzed in a subsequent batch.

2.2.3 *Analysis*

Water chemistry tabulation is pending; however Cu concentrations were confirmed daily to be within 2.5% of target concentrations each day of the experiment. Total abundance, mayfly abundance (E), stonefly abundance (P), caddisfly abundance (T), dipteran abundance (D), and EPT abundance were tabulated for the 96 hr survival sample and the eight drift samples (12, 24, 36, 48, 60, 72, 84, and 96 hr) for each of the sixteen experimental units. Additionally these measures were tabulated for the three Day-0 samples. Mortality for each experimental unit was estimated as the remainder after subtracting total drift and survival from the average of the three Day-0 samples. Usefulness of counts, proportions of Day-0 samples and proportions of the average of all controls will be compared.

Colorado and National policy relies on LC_{50} values to derive acute standards. If observed in sufficient abundance, 96 hr LC_{50} values for each taxon or group of taxa were calculated using the dose response model function ("drm") in package 'drc' (Ritz et al. 2015) in Program R. DRC allows a suite of models to be applied to proportion and total counts data representing survival, mortality or effect data. The models with the best parsimonious fit (AIC) were then used to supply LC_{50} or EC_{50} values (EC_5, EC_{10} and EC_{20} were also calculated as these are used by regulators abroad). Comparison of treatment levels to the control was made using Dunnett's Test and comparisons between treatment levels were made using REGWQ (Ryan-Einot-Gabriel-Welsch-Quiot) multiple comparison within ANOVA. The unbalanced replication at higher treatment levels was advantageous for effect concentration modeling but prevents multiple comparisons in ANOVA at the 17 and 35 µg/L treatment group. Modeling the timing of drift (12, 24, 36, 48, 60, 72, 84, and 96 hr or day/night) as a function of concentration, feeding guild, life history, or taxonomic group is pending.

2.3 Results

The acute copper standard for the State of Colorado is 7.0 µg/L when adjusted for our observed hardness of 50 µg/L. Total (cumulated) drift of macroinvertebrates was greater in most treatments than in controls. This includes a statistically significant difference between the 2.24 µg/L (lowest) treatment and the control (p=0.02). This trend was observed in Ephemeropteran, Plecopteran, and Trichopteran taxa (EPT). After removal of drifting organisms Total Macroinvertebrates counts showed a clear dose response (Figure 2.1) with a statistically significant difference between each treatment group and control (p<0.001 for each). This includes the 2.24 µg/L (lowest) treatment. Comparison to the 35 and 17 µg/L treatment levels is not reported due to insufficient replication.

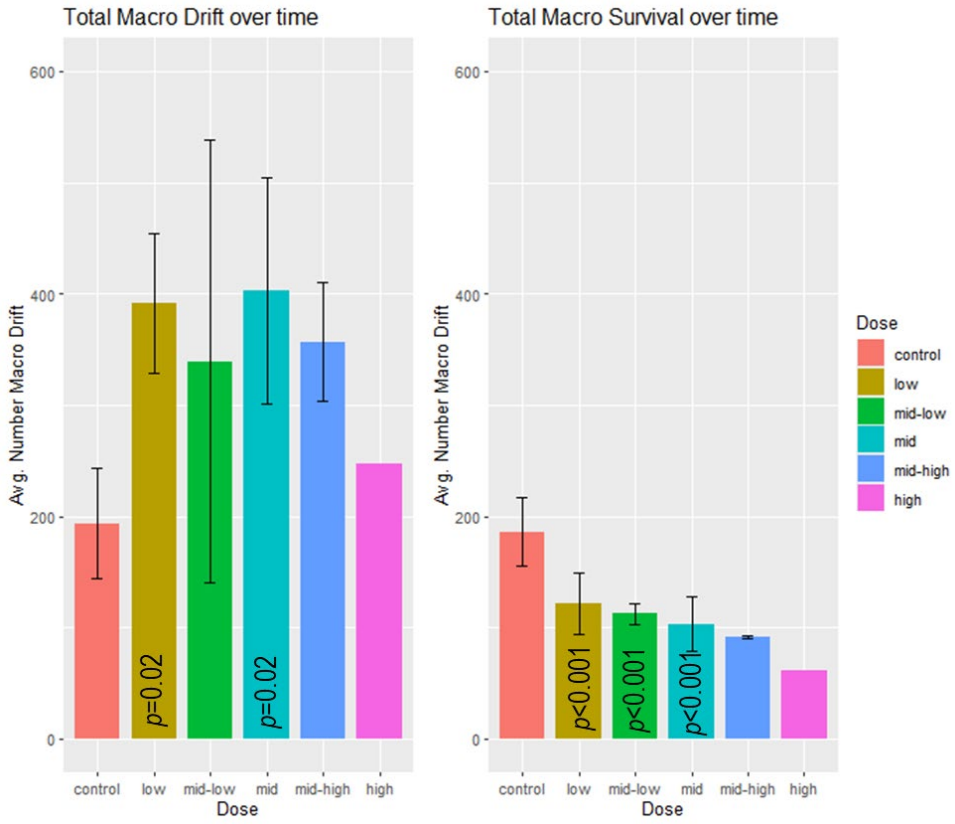


Figure 2.2 Total macroinvertebrate drift and survival. Drift of aquatic insects (left) was generally greater in treatments than in control. Drift in higher concentrations may have been reduced by mortality. Survival (right) shows a clear dose response relationship with controls having significantly more survivors after 96 hrs than any other treatment. Treatment groups that are significantly different from the controls are designated with a significant *p*-value.

Analysis of individual species and effect concentrations (LC_{50}) is pending. Even after acclimation for 48 hours prior to exposure to the toxicant, drift appeared greatest in the first 24-48 hours (Figure 2.2). Also, a possible day-night effect was observed. Analysis of these for feeding guilds, life histories, and specific taxa is pending. Such findings might someday explain the limited distribution of tolerant invertebrates.

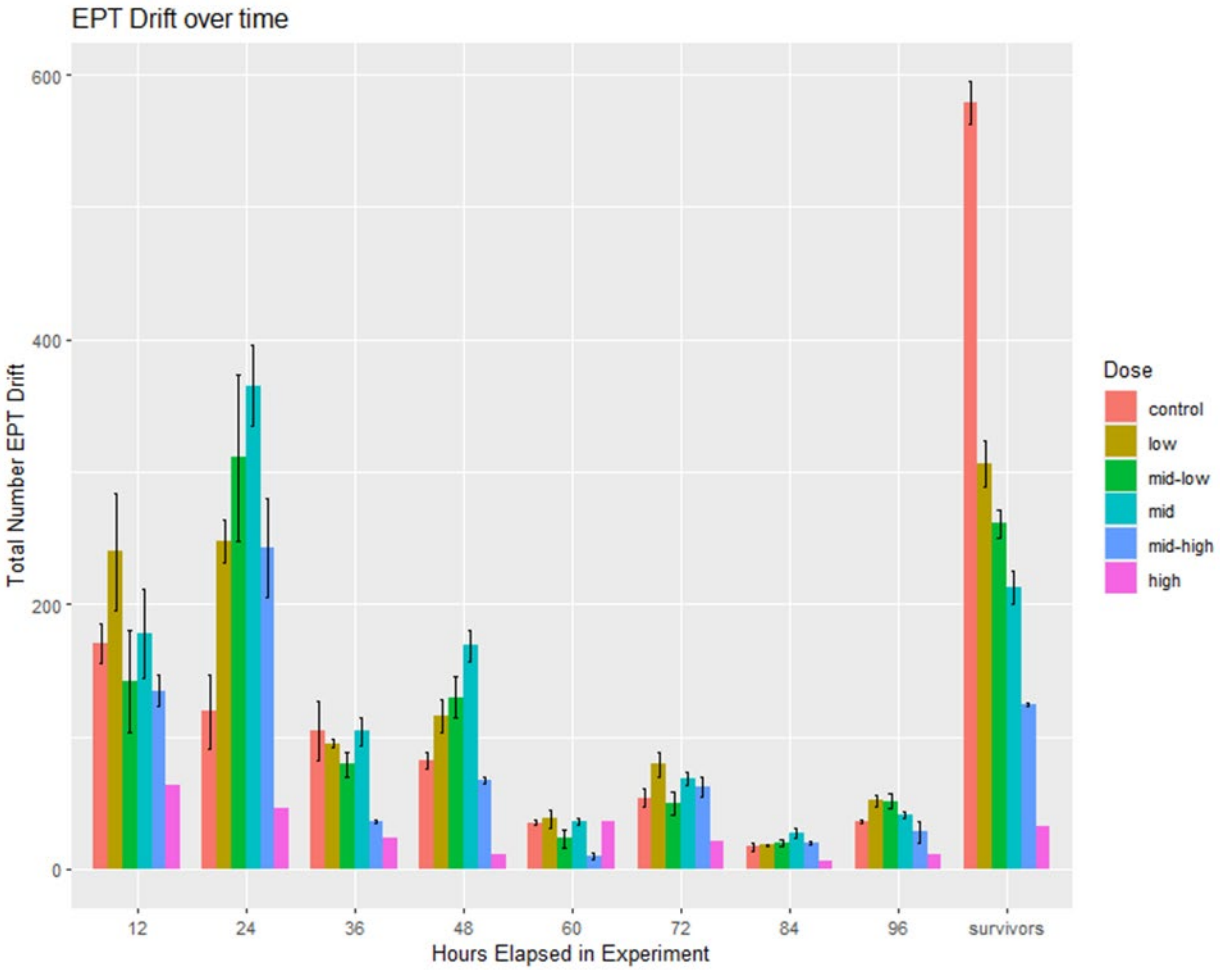


Figure 2.3 Ephemeroptera, Plecoptera, and Trichoptera (EPT) drift and survival over eight 12-hour sampling periods – Drift at day-vs-night or early vs later in the 96 hr period could be explained by life history (in example free living vs net spinning), feeding guild, drift propensity, or sensitivity. Statistical analysis pending.

2.3 References

Besser, J. M. and K. J. Leib. 2007. Toxicity of metals in water and sediment to aquatic biota. In Integrated Investigations of Environmental Effects of Historical Mining in the Animas River Watershed, San Juan County, Colorado, vol. 1, pp. 839-846. U.S. Geological Survey.

Buchwalter, D., W. H. Clements, and S. Luoma. 2017. Modernizing water quality criteria in the United States: A need to expand the definition of acceptable data. Environmental Toxicology and Chemistry 36(2): 285-291.

Clements, W. H. 2004. Small-scale experiments support causal relationships between metal contamination and macroinvertebrate community responses. Ecological Applications 14(3): 954-967.

Clements, W. H., C. Hickey, and K. Kidd. 2012. How do aquatic communities respond to contaminants? It depends on the ecological context. Environmental Toxicology and Chemistry 31(9): 1932-1940.

Clements, W. H. and C. Kotalik. 2016. Effects of major ions on natural benthic communities: An experimental assessment of the U.S. Environmental Protection Agency aquatic life benchmark for conductivity. Freshwater Science 35(1): 126-138.

Duggan, S. B. and C. J. Kotalik. 2020. Not the latest science: National recommended water quality criteria for aquatic life under the Clean Water Act. Minnesota Journal of Law, Science & Technology 21: 371. Available at: https://scholarship.law.umn.edu/mjlst/vol21/iss2/4

Environmental Protection Agency. 1985. Guidelines for deriving numerical national water quality criteria for the protection of aquatic organisms and their uses. pp. 1–59. Office of Water Regulations and Standards, Criteria and Standards Division, Washington, D.C.

Clements, W. H., P. Cadmus, and S. F. Brinkman. 2013. Responses of aquatic insects to Cu and Zn in stream microcosms: Understanding differences between single species tests and field responses. Environmental Science and Technology 47(13): 7506-7513.

Clements, W. and J. Rohr. 2009. Community responses to contaminants: Using basic ecological principles to predict ecotoxicological effects. Environmental Toxicology and Chemistry 28(9): 1789.

Ford, A., M. Ågerstrand, B. W. Brooks, J. Allen, M. G. Bertram, T. Brodin, Z. Dang, S. Duquesne, R. Sahm, F. Hoffman, H. Hollert, S. Jacob, N. Klüver, J. M. Lazorchak, M. Ledesma, S. D. Melvin, S. Mohr, S. Padilla, G. G. Pyle, S. Scholz, M. Saaristo, E. Smit, J. A. Steevens, S. van den Berg, W. Kloas, B. B. M. Wong, M. Ziegler, and G. Maack. 2021. The role of behavioral ecotoxicology. Environmental Science & Technology 55(9): 5620-5628.

Iwasaki, Y. and W. Clements. 2015. A continuous need to determine what we should protect in ecological risk assessments. Environmental Science & Technology 49(13): 7520-7521.

Iwasaki, Y., T. Schmidt, and W. Clements. 2018. Quantifying differences in responses of aquatic insects to trace metal exposure in field studies and short-term stream mesocosm experiments. Environmental Science & Technology 52(7): 4378-4384.

Moe, S., K. De Schamphelaere, W. Clements, M. Sorensen, P. Van den Brink, and M. Liess. 2012. Combined and interactive effects of global climate change and toxicants on populations and communities. Environmental Toxicology and Chemistry 32(1): 49-61.

Scott, G. and K. Sloman. 2004. The effects of environmental pollutants on complex fish behaviour: Integrating behavioural and physiological indicators of toxicity. Aquatic Toxicology 68(4): 369-392.

Sprague, J. B. 1971. Measurement of pollutant toxicity to fish—III. Water Research 5(6): 245-266.

Stephan, C. E., D. I. Mount, D. J. Hansen, J. R. Gentile, G. A. Chapman, and W. A. Brungs. 1985. Guidelines for deriving numerical standards for the protection of aquatic organisms and their uses. U.S. Environmental Protection Agency, Springfield, VA. PB85-227049.

Establishing Temperature Tolerance Ranges for Fish Species through Electrocardiogram Analysis

Dr. Tawni B. R. Firestone, Riley Dils (CSU)

3.1 Introduction

Water temperature is one of the most important abiotic factors contributing to fish survival. Unfortunately, most fish species are poorly able to adapt to variable water temperatures outside of their normal annual or seasonal changes. Streams used by many fish species across Colorado are experiencing variable water temperatures of high and low extremes and extreme rates of change and succumb to low survival. Tolerant temperature ranges for fish species are dependent on different life stages of fish where larval fish and adult fish of the same species may exhibit large variations in tolerant temperature ranges. By using acute and chronic laboratory temperature tests with adult fish, we will be able to define field-based temperature tolerance ranges for native fish species in Colorado.

Acute and chronic laboratory temperature tests have been used to develop protective temperature standards, but these focus on larval fish stages and do not allow for the testing of adult fish. Laboratory tests have included critical thermal maximum (CTMax) and minimum (CTMin) trials, and upper and lower incipient tests with larval fish and are commonly used to establish a tolerance temperature range (Beitinger et al. 2000). Unfortunately, these tests involve a small amount of water that is increased (CTMax) or decreased (CTMin) by 0.3°C min^{-1} (18°C hr^{-1}) which is not easily achievable for large tanks of water that we would need to use with adult fish nor can these type of tests be conducted in the field.

The Colorado Water Quality Control Commission Policy 06-1 (WQCC 2011) allows for other testing methods to determine physiological endpoints to establish temperature criteria for fishes. The optimal temperature ranges can be derived from growth rate, digestion rate, swimming performance, gross conversion efficiency, metabolic rate, and cardiac rate over a range of temperatures. Measuring cardiac output is an efficient method to establish tolerant temperature ranges for adult fish because as the water temperatures increase, the increased metabolic rate of the fish will require the fish to transfer more oxygen to the tissues. Since the arterial blood is fully saturated with oxygen, by increasing the cardiac output more oxygen can be supplied to the tissues. Thus, any environmental change that alters oxygen uptake in the fish also involves a significant change in cardiac output, which can be measured with an ECG. One change includes cardiac arrhythmias. The exposure of the fish to extreme temperature change can result in life-threatening cardiac arrhythmias (Farrell 2009).

This report summarizes the research conducted to optimize the use of an ECG for assessing cardiac responses in adult Rainbow Trout (*Oncorhynchus mykiss*) and adult White Suckers (*Catostomus commersonii*) in relation to heat tolerance. The study with Rainbow Trout was conducted at the CPW Toxicology Laboratory using six fish and the study with White Suckers was conducted at Watson Lake in Bellvue, CO with nine fish. Specifically, this report outlines a series of trials conducted to optimize ECG measurements using Rainbow Trout and White Suckers as a surrogate using AQUI-S 20E for anesthesia for future use with Flannelmouth

Sucker, Bluehead Sucker, and Roundtail Chub. The trials assessed the effects of light sedation, anesthesia duration, and water temperature on cardiac function, with a focus on refining techniques for future research.

3.2 Methods

3.2.1 *Rainbow Trout Trials*

Our first objective was to evaluate the efficacy of AQUI-S 20E as an anesthetic agent for conducting cardiac measurements in Rainbow Trout and determining the most effective concentration. We also used this time to optimize the ECG electrodes with the fish. Six adult Rainbow Trout from the CPW Fish Research Hatchery were used in various trials to optimize ECG recording methods and assess the physiological responses to sedation and temperature changes.

One 100 L glass aquarium was used with a polyurethane ECG chamber suspended inside of the tank to fill the chamber half way with the water in the aquarium (Figure 3.1). The aquarium contained up to 70 L of dechlorinated water with AQUI-S 20E solution added during each trial. Fully anesthetized trout were placed ventral side up in a custom designed holder in an individual ECG chamber to support the fish and water was circulated from a larger tank with AQUI-S 20E into the ECG chamber to allow for circulation throughout the system. The ECG chamber contained holes throughout the chamber to allow for flowing water and was placed inside the same tank the fish was anesthetized in. The fish remained in the chamber, ventral side up and gills completely submerged in the water containing AQUI-S 20E solution for the duration of the test. Three electrodes were placed on the ventral surface with adhesion gel located above and below the heart ventricle and one in the middle as a grounding electrode. Adjustments of electrode placements were made until distinct P, Q, R, S, and T waves are formed as seen on the iWorx® LabScribe program.

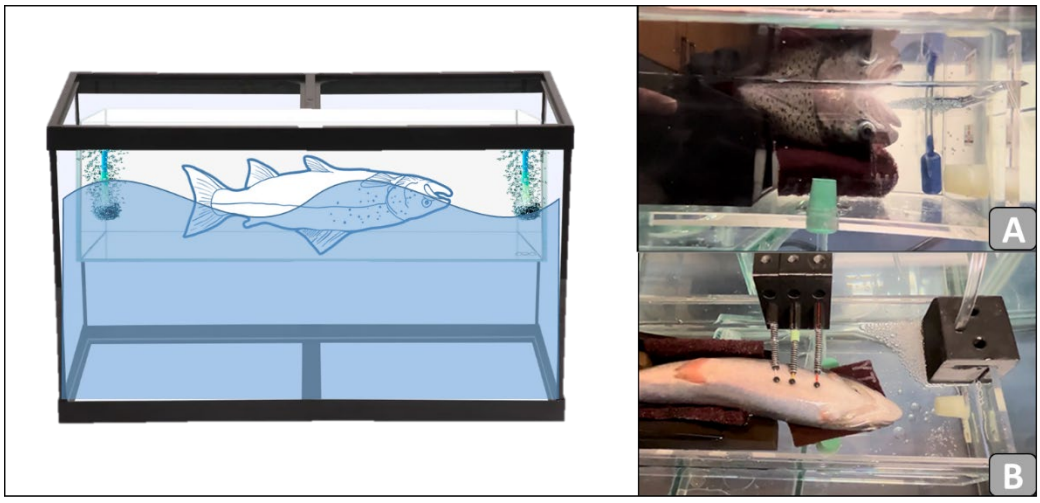


Figure 3.1 Initial system setup with ECG chamber suspended in a 100 L glass aquarium. (A) Placement of fish in the ECG chamber with the placement of electrodes (B) with a positive electrode (red), grounding (green), and negative (white).

3.2.2 *Anesthetic Protocol for Rainbow Trout*

For initial trials, a concentration of 15 ppm of AQUI-S 20E was used but then decreased to 13.95 ppm by diluting 8.69 mL of the solution in 70 L of dechlorinated water. Water temperature was monitored, starting around 14.5°C for each trial. Each trial included a monitoring phase where fish response to anesthesia was recorded. Fish remained anesthetized for up to one hour for proper readings. Fish were recovered in large tanks with fresh water and aeration to ensure they could recover and were then euthanized with tricaine methanolsulfate (MS-222) and the end of the study.

Trials continued with six total fish, focusing on variations in size and weight of the fish and modifying ECG chamber setups. Each fish was monitored closely throughout the process, with anesthesia and recovery times documented. Despite achieving full anesthesia in all cases, technical difficulties such as interference from heating equipment in later trials prevented successful ECG recordings.

3.2.3 *White Sucker Trials*

This section details the methodology and findings from trials conducted to optimize the use of AQUI-S 20E for anesthesia in adult White Suckers in the field during ECG and temperature assessments. The study focused on the administration of varying doses of AQUI-S 20E to use with adult suckers, controlling for a temperature increase in the ECG chamber, and the effectiveness of aeration in maintaining dissolved oxygen levels.

3.2.4 *Temperature Trial Setup*

The experimental setup consisted of four interconnected systems: a heating tank, an ECG chamber, a mock tank, and a collection tank (Figure 3.2). The heating tank, a 75 L polyethylene container, held 49.85 L of lake water and was equipped with a 1000-watt heater controlled by a B-Series Love temperature controller to maintain a consistent temperature of 43°C. An air stone was added to aerate the water in the heating tank. The mock tank contained 3 L of lake water and was designed to minimize electrical interference in the ECG chamber. Two pumps were submerged in the heating tank, controlled by the temperature controller, and pumped hot water to both the ECG chamber and the mock tank at a rate of 250 mL min^{-1}, with a temperature increase of 0.3°C min^{-1}. The ECG chamber also contained 3 L of lake water, suspended above a larger aquarium tank. This chamber was designed to accommodate a single fish secured in a 3D-printed holder, with a damp paper towel covering the posterior end of the fish to ensure moisture retention. The setup included two airstones for aeration and five holes for water to flow through the ECG chamber, into the aquarium and into a collection bucket.

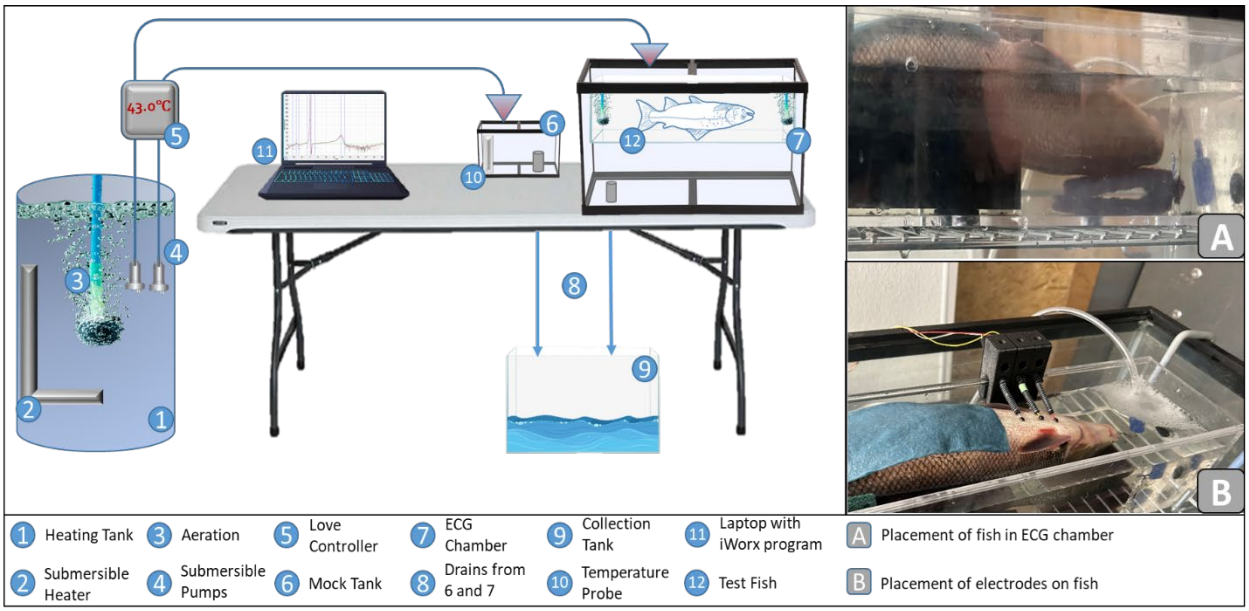


Figure 3.2 Diagram of ECG system setup with the heating tank (1), mock tank (6), the ECG chamber (7), and a collection tank (9). (A) Placement of fish in the ECG chamber with wet towel placed on the fish and the placement of electrodes (B) with a positive electrode (red), grounding (green), and negative (white).

3.2.5 *Anesthetic Protocol for White Suckers*

Two sedation levels were used with White Suckers because we anticipated these fish to respond differently to the AQUI-S 20E compared to the Rainbow Trout. A 75 L glass aquarium was separate from the ECG system and was used for the initial (high) sedation up to 10 minutes. The initial sedation started at 100 ppm and was lowered throughout the trials, as described below.

A lighter sedation was maintained throughout the ECG system after the initial high sedation of the fish. A total of 7.34 mL of AQUI-S 20E was added to 55.8 L of water (total system volume), maintaining an initial low concentration of 14.79 ppm. Specifically, 6.54 mL was added to the heating tank (49.8 L of water), 0.40 mL to the ECG chamber (3 L), and 0.40 mL to the mock tank (3 L). Following this, the system was mixed by turning on the pumps for 20 minutes to ensure uniformity, and the collection tank was emptied back into the heating tank to maintain concentration levels. The ECG chamber and mock tank were refreshed with lake water and a new dose of AQUI-S 20E for each trial.

3.2.6 *ECG System and Temperature*

Trials were conducted in a mobile laboratory parked next to Watson Lake located in Bellvue, CO. Fish were collected by setting four gill nets using a motorized boat. Nets were pulled after one hour to ensure fish were not injured. Fish were held in a net pen in the lake until the start of each individual trial.

Temperature trials were conducted following critical thermal maximum procedures by Becker and Genoway (1979). B-Series Love temperature controllers were connected to pumps that

pumped water from the heating tank to both the mock tank and ECG chamber. The controllers were programmed to change the water temperature + 0.3°C min^{-1}. Pre- and post-trial temperatures were recorded with a Traceable Lollipop thermometer that was calibrated before each test. Each trial lasted until a heart arrhythmia was detected with the ECG and iWorx LabScribe system. A heart arrhythmia was defined as an atrioventricular block which is the inability of the action potential to progress from the atrium to the ventricle (Vornanen et al. 2024).

3.3 Results

3.3.1 *Rainbow Trout Trials*

The following trial results are reported focusing on light sedation use of AQUI-S 20E, anesthesia duration, and water temperature with adult Rainbow Trout (Table 3.1).

Trial 1 – In the first trial, a Rainbow Trout measuring 281 mm and 269 g was sedated with 15 ppm AQUI-S 20E in 14.2°C water. Initial observations included normal gill movements. After ten minutes, the fish displayed reduced gill activity and decreased responsiveness. At the 20-minute mark, the fish was gilling even slower and the AQUI-S concentration was reduced by adding 5 L of freshwater to the tank. The fish was subsequently moved to the ECG chamber for cardiac measurements. The fish remained in the chamber for 40 minutes before being transferred to a recovery tank. No arrhythmia was detected. Average T waves were 33.71 $\pm$ 4.5 mV and average heart rate was 51 $\pm$ 7 bpm. Recovery was observed after eight minutes.

Trial 2 – A second fish (258 mm, 216 g) was anesthetized in a similar manner but at 13.95 ppm (8.69 mL of AQUI-S 20E in 70 L of dechlorinated water) and in 14.3°C water. Following ten minutes of anesthesia, ECG electrodes were applied, but insufficient electrode size and excessive interference compromised data collection. After 41 minutes of anesthesia, the fish was removed and recovered after six minutes. No cardiac measurements were collected.

Trial 3 – Another fish (282 mm, 264 g) was sedated with 13.95 ppm in 14.5°C water. Three minutes into the test, the fish flipped over, was using its tail fin in an attempt to swim, and was gilling at a normal rate. Eleven minutes in to the initial sedation the fish was completely flipped over, gilled slowed, and the fish was not responsive. The fish was moved into the ECG chamber. Smaller ECG electrodes were placed on the ventral side of the fish with adhesion gel. After 40 minutes the fish was removed and placed into a recovery tank. No arrhythmia was detected but inference with the electrodes interfered with the successful detection of the heart rate. The fish fully recovered after six minutes.

Trial 4 – A Rainbow Trout (270 mm, 214 g) was placed into a 13.95 ppm light sedation at 14.4 °C. The fish was fully anesthetized after 16 minutes and then placed into the ECG chamber. ECG electrodes were placed on the fish and the fish remained in the chamber for the duration of the test for 54 minutes. No arrhythmia was detected but inference with the electrodes interfered with the successful detection of the heart rate. The fish fully recovered after four minutes.

Trial 5 – To investigate the influence of temperature when using AQUI-S 20E, a fish (262 mm, 221 g) was anesthetized while water temperature was incrementally raised by 0.2°C from 15.3°C to 19.8°C over 30 minutes. Unfortunately, the fish succumbed to mortality likely due to reaching a lethal temperature and not due to the use of the AQUI-S with an increase in temperature.

Trial 6 – The last trial was used to determine ECG efficacy in a smaller tank size and after adding a temperature component. The Rainbow Trout (279 mm, 272 g) was anesthetized in 13.95 ppm AQUI-S 20E solution (5.72 mL of AQUI-S 20E solution in 46 L of dechlorinated water). The fish was fully anesthetized after 10 minutes and placed into the ECG chamber. Electrodes were placed on the ventral side of the fish. The water temperature started at 14.4°C. Programmable B-Series Love Controllers were connected to aquarium heaters to control the rate of temperature increase. Unfortunately, the heaters and temperature controllers interfered with the ECG readings. Thus, we could not measure the effects of temperature on the fish. The fish remained in the chamber during the trial for 60 minutes. The fish was recovered fully after 4 minutes.

Table 3.1 Summary of AQUI-S 20E solution trials for Rainbow Trout

Species	Length (mm)	Weight (g)	Water Temperature (°C)	Sedation Level	AQUI-S 20E Dose	Anesthetized Time	Recovery Time
Rainbow Trout	281	216	14.2	Low	15 ppm	40 min	8 min
Rainbow Trout	258	216	14.3	Low	13.95 ppm	41 min	6 min
Rainbow Trout	282	264	14.5	Low	13.95 ppm	40 min	6 min
Rainbow Trout	270	214	14.4	Low	13.95 ppm	54 min	4 min
Rainbow Trout	262	221	15.3 – 19.8	Low	13.95 ppm	30 min	Mortality
Rainbow Trout	279	272	14.4	Low	13.95 ppm	60 min	4 min

3.3.2 *White Sucker Trials*

The following trial results are reported focusing on two sedation levels using AQUI-S 20E, anesthesia duration, increasing the water temperature in the ECG chamber, and detections of an arrhythmia during temperature trials with adult White Suckers (Table 3.2).

Trial 1 – The first White Sucker measured 365 mm and weighed 557 g. A high sedation dose of 100 ppm was administered in a 75 L glass aquarium using 10.72 mL AQUI-S 20E in 12.05 L of lake water. The temperature of the tank was 24.7°C (lake temperature). After one minute and 30 seconds, the fish was unresponsive and subsequently transported to the ECG chamber. A low sedation level of 14.79 ppm was maintained throughout the ECG system during the trial. The temperature of the system started at 24.5°C. The fish remained in the ECG chamber, ventral side up and gills completely submerged in the water containing AQUI-S 20E solution for the duration of the test. ECG electrodes were placed on the ventral side of the fish with adhesion gel during the test. Water from the heating tank was pumped into the ECG chamber at +0.3°C min^{-1}. The fish was removed after 17 minutes because the temperature controllers were not functioning properly. The fish fully recovered after 10 minutes. No cardiac measurements were collected.

Trial 2 – The second White Sucker measured 464 mm and weighed 1,327 g. An initial high sedation dose was adjusted to 92.3 ppm and temperature of the tank was 25.6°C. After 2 minutes and 20 seconds, the fish was transported to the ECG chamber (25.4°C) with a low sedation level of 14.79 ppm. The trial proceeded with the ECG setup, but the fish was removed after 18 minutes due to labored gilling. Recovery took nine minutes. No cardiac measurements were collected. Dissolved oxygen levels were measured after at the end of the test, and were measured at 55%. A new aeration system was built for the next trial to include aeration in the heating tank and an airstone in the ECG chamber.

Trial 3 – Aeration was supplied to the ECG chamber with one airstone to ensure the DO levels in the chamber remained above 80% during each trial. The fish (382 mm, 707 g) in this trial was used as a control fish to ensure the fish could withstand 14.79 ppm of AQUI-S 20E for one hour with the new aeration system.

The high sedation was reduced again to reach 80.05 ppm AQUI-S 20E (10.72 mL of AQUI-S 20E in 15.05 L of lake water). The temperature of the high sedation tank was 21.1°C. After two minutes into the high sedation period, the fish completely flipped over, gilled slowly, and the fish was not responsive. The fish was then transferred to the ECG chamber. The DO in the ECG chamber started at 99.9% and the temperature started at 21°C. The ECG setup maintained a light sedation for up to one hour of 14.79 ppm connected to the ECG machine. The fish was removed after one hour and no irregular heart rate was detected. At the end of the trial, the DO in the tank was 86.2% and the temperature of the tank was 23°C. The fish fully recovered in a tank filled with oxygenated lake water after 17 minutes. No arrhythmia was detected and average T wave amplitudes were 76.97 mV and average heart rate was 66 $\pm$ 32 bpm.

Trail 4 – The fourth White Sucker measured 508 mm and 1,547 g. The initial high sedation was lowered to 70.7 ppm since the previous fish were still flipping early in previous trials. The temperature of the high sedation tank was 25.3°C. After 7 minutes in the initial sedation, the fish completely flipped over, gilled slowly, and the fish was not responsive. The ECG setup maintained a light sedation for up to one hour with 14.79 ppm of AQUI-S 20E in lake water. The initial temperature of the ECG chamber was 21°C and the DO was 104.6%. The fish remained in the ECG chamber for 13 minutes but was then removed because the temperature controllers were not working properly. No cardiac measurements were collected. The fish recovered fully after 22 minutes and 15 seconds in fresh lake water.

Trial 5 – A White Sucker (411 mm, 916 g) was placed into a high sedation tank (70.7 ppm). Temperature and DO measured 21.9°C and 106.8%, respectively. The fish was transported to the ECG chamber after 6 minutes. The fish was gilling very slowing in the ECG chamber, thus we decided to end the trial and place the fish in the recovery tank. No cardiac measurements were collected. The fish recovered after 4 minutes.

Trial 6 – The White Sucker (382 mm and 702 g) was placed into the high sedation tank at 48.1 ppm. The temperature of the tank was 22.2°C and DO was 100%. After 8 minutes the fish was transported to the ECG chamber with the low sedation. The initial temperature of the ECG chamber was 22.5°C with a DO of 101.1%. A temperature trial was conducted by increasing the temperature in the ECG chamber by + 0.3°C. The fish remained in the ECG chamber for 40

minutes and was removed after detecting a heart arrhythmia. Average T wave amplitudes were 49.89 mV and average heart rate was 92 $\pm$ 8 bpm. The final temperature of the tank was 30°C and the DO was 52%. The fish recovered after 5 minutes.

Trail 7 – The White Sucker (412 mm, 874 g) was used as a control fish to ensure the fish could withstand the 14.79 ppm of AQUI-S 20E for one hour with an additional airstone added to the ECG chamber. A high sedation of 48.1 ppm was used and the temperature of the high sedation tank was 19.6°C and DO measured 106%. The fish was moved to the ECG chamber after 8 minutes. Sedation in the ECG chamber remained at a low dose. DO in the ECG chamber measured 100% and the temperature of the chamber was 19.9°C. The fish was removed after one hour and no irregular heart rate was detected. Average T wave amplitudes were 77.98 mV and average heart rate was 55 $\pm$ 4 bpm. The final temperature of the chamber was 20.4°C and the DO was 75.6 %. The fish recovered fully after 5 minutes

Trial 8 – The White Sucker (504 mm, 1,439 g) was placed into the high sedation tank (48.1 ppm; temperature: 21.8°C, DO: 104.5%). After 7 minutes the fish was transported to the ECG chamber. The DO and temperature in the ECG chamber measured 115% and 21.9 °C, respectively. The system maintained a light sedation (14.79 ppm). The fish was removed from the system after 22 minutes because the temperature controllers were not functioning properly. No cardiac measurements were collected. The fish was fully recovered after 24 minutes.

Trial 9 – The White Sucker (476 mm, 1,277 g) was placed into the high sedation tank (48.1 ppm; temperature: 20.5°C; DO 112%) and moved to the ECG chamber after 8 minutes. The DO in the ECG chamber (low sedation) was 110% and the temperature of the ECG chamber started at 20.9°C. The fish remained in the ECG chamber for the duration of the trial. A temperature trial was conducted by increasing the temperature by +0.3°C min^{-1}. The fish was removed after 43 minutes after an arrhythmia was detected. Average T wave amplitudes were 30.21 mV and average heart rate was 90 $\pm$ 25 bpm. The final temperature was 32.2°C and the DO measured at 90.2%. The fish fully recovered after 4 minutes.

Table 3.2 AQUI-S, ECG setup, and temperature trial results for adult White Suckers. Dashes indicate no data collected due to a failure in the trial.

Trial	Species	Length (mm)	Weight (g)	High Sedation (ppm)	Low Sedation (ppm)	Start Temp (°C)	End Temp (°C)	Start DO (%)	End DO (%)	Arrhythmia Detected	Trial Time	Recovery Time
1	White Sucker	365	557	100	14.79	24.5	24.5	–	–	–	17 min	10 min
2	White Sucker	464	1,327	92.3	14.79	25.6	25.6	–	55	–	18 min	9 min
3	White Sucker	382	707	80.05	14.79	21.0	23.0	99.9	86.2	No	60 min	17 min
4	White Sucker	508	1,547	70.7	14.79	21.0	–	104.6	–	–	13 min	22 min
5	White Sucker	411	916	70.7	14.79	21.9	–	106.8	–	–	–	4 min
6	White Sucker	382	702	48.1	14.79	22.5	30.0	101.1	52	Yes	40 min	5 min
7	White Sucker	412	874	48.1	14.79	19.9	20.4	100	75.6	No	60 min	5 min
8	White Sucker	504	1,439	48.1	14.79	21.9	21.9	115	–	–	22 min	24 min
9	White Sucker	476	1,277	48.1	14.79	20.9	32.2	110	90.2	Yes	43 min	4 min

3.3.3 *ECG Reading Summary*

In summary, we investigated the effects of AQUI-S20E sedation doses on Rainbow Trout and White Suckers for current and future temperature trials. Rainbow Trout were subjected to light sedation at 13.95 ppm AQUI-S 20E for up to one hour. Heart rate measurements were successfully obtained from only one fish, yielding a rate of 51 ± 7 bpm (Table 3.3). Recovery from sedation in Rainbow Trout took between 4 to 8 minutes. White Suckers required a two stage sedation process: initially a heavy sedation with 48.1 ppm AQUI-S 20E for a duration of up to 10 minutes, followed by a transition to a low sedation level of 14.79 ppm for an additional hour.

Table 3.3 Average resting, maximum, and minimum heart rates (bpm) and average T wave amplitude (mV) for control and temperature trial White Suckers.

Trial	Species	Resting HR (Avg)	Max HR	Min HR	Δ Temperature	T Wave Amplitude (Avg)	Trial Type
3	White Sucker	66 ± 32	107	20	+2.0°C	76.97 mV	Control
7	White Sucker	55 ± 4	66	45	+0.5°C	77.98 mV	Control
6	White Sucker	92 ± 8	136	42	+7.5°C	49.89 mV	Arrhythmia
9	White Sucker	90 ± 25	123	42	+11.3°C	30.21 mV	Arrhythmia

The average resting heart rates observed in control fish were 60.5 bpm, while temperature trial fish exhibited an average heart rate of 90.0 bpm. Arrhythmia was recorded in both temperature trial fish after 42 and 45 minutes, corresponding to final temperatures in the ECG chamber of 30°C and 32.2°C, respectively. Notably, as temperature increased, heart rates initially rose then fell or remained steady, while T Wave amplitude (mV) indicated a consistent linear decrease before an arrhythmia was detected (Figure 3.3).

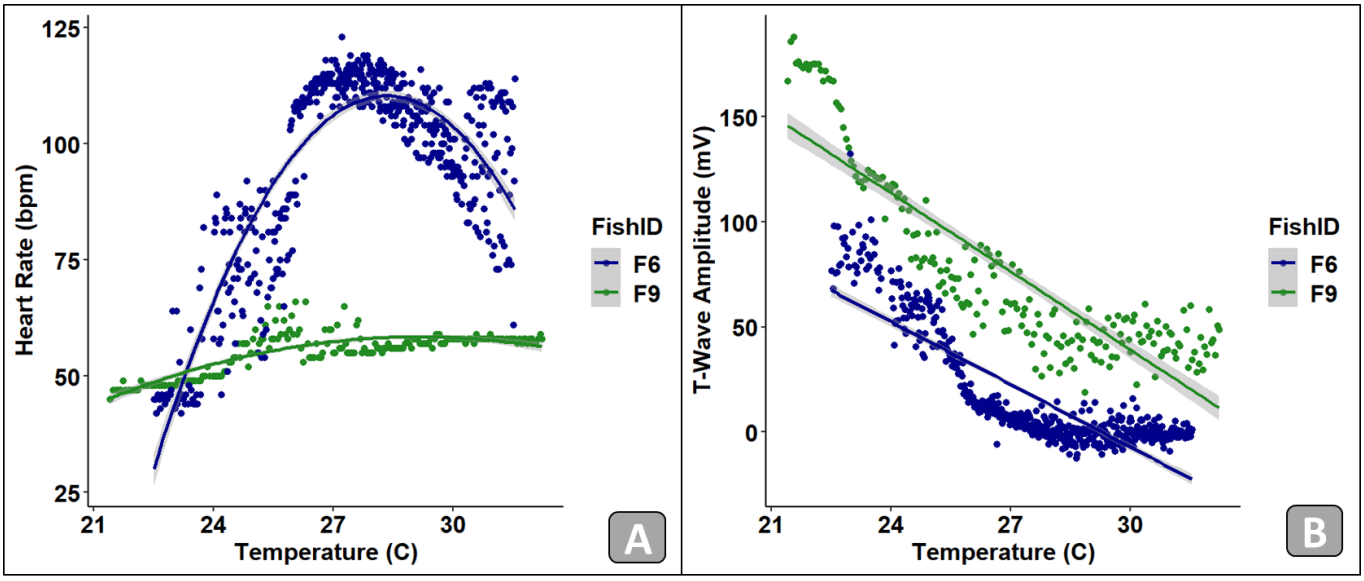


Figure 3.3 A) Heart rate (bpm) for temperature trial fish numbers 6 (blue) and 9 (green) as temperature increased in the ECG chamber. B) T-Wave amplitude for temperature trial fish as temperature increased.

3.4 Discussion

In total, five Rainbow Trout were anesthetized across initial trials, with consistent findings regarding the anesthetic's efficacy when using 13.95 ppm AQUI-S 20E for up to one hour. Recovery times varied slightly, averaging between three to eight minutes post-anesthesia. However, the presence of interference during the ECG measurements highlighted a significant challenge in obtaining reliable cardiac data in the presence of temperature controllers.

Trials for the nine White Suckers indicate that a high sedation dose of 48.1 (10.72 mL AQUI-S 20E in 25.05 L of water) for up to 8 minutes, followed by a light sedation of 14.79 ppm (7.34 mL AQUI-S 20E in 55.8 L of water) for up to one hour are the best sedation methods for our experiment. Our methods show that these sedation levels are effective for anesthetizing adult suckers for ECG measurements and temperature trials. Aeration and careful monitoring of temperature and oxygen levels are essential for maintaining fish health during the experiment. Future studies should focus on further optimizing these conditions and addressing equipment reliability to improve data quality.

Resting heart rates for both Rainbow Trout and White Suckers ranged from 50 to 70 bpm. In White Suckers, heart rates increased with rising water temperatures, as expected, up to 91 bpm. This is due to the enhanced metabolic demands associated with higher temperatures, which require fish to transport more oxygen to their tissues (Neubauer and Andersen 2019). Thus, changes in temperature affect oxygen uptake by increasing their heart rate. We also noted differences in T wave amplitudes between the control and temperature trial White Suckers. Average T wave amplitude for control fish averaged 77.48 mV and 39.55 mV for temperature trial fish. Low T wave amplitudes in ECGs are critical markers for assessing cardiac health (Duca et al. 2023; Zena et al. 2024). Specifically, reduced T wave amplitudes can contribute to the development of cardiac arrhythmias. The T wave reflects the repolarization of the ventricles; therefore a decrease in amplitude may indicate abnormalities in the repolarization process of the heart as the heart struggles to maintain normal electrical activity (Kenny and Brown 2022). Thus, the increasing temperatures during the experiment likely impaired the blood flow to the heart, altering the repolarization process, and resulting in diminished T wave amplitudes and the detected arrhythmias. Measuring heart rate, T wave amplitudes, and detecting arrhythmias can be a good indicator of thermal stress. Therefore, we can use the methods listed here to determine maximum temperatures adult native fish can tolerate.

3.5 Conclusion

Our series of trials establishes a foundational protocol for future cardiac measurements during temperature trials for adult Suckers using AQUI-S 20E for anesthesia. Further refinements in electrode selection, placement, and using more replicates of control fish will be essential to enhance data quality in future research projects. Continued research understanding temperature effects on cardiac function in fish is needed, given the critical implications for developing temperature standards for adult native fish species.

3.6 References

Becker, C. D., and R. G. Genoway. 1979. Evaluation of the critical thermal maximum for determining temperature tolerance of freshwater fish. Environmental Biology of Fishes 4: 245-256.

Beitinger, T. L., W. A. Bennett, and R. W. McCauley. 2000. Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. Environmental Biology of Fishes 58: 237-275.

Duca, S. T., M. Roca, A. D. Costache, A. Chetran, I. Afrăsânie, R. Miftode, I. Tudorancea, I. Matei, R. Ciorap, O. Mitu, M. C. Badescu, D. Iliescu-Halitchi, C. Iliescu, F. Mitu, L. Catalina, and I. I. Costache. T-wave analysis on the 24 h Holter ECG monitoring as a predictive assessment of major adverse cardiovascular events in patients with myocardial infarction: A literature review and future perspectives. Life 13(5): 1155.

Farrell, A. P., E. J. Eliason, E. Sandblom, and T. D. Clark. 2009. Fish cardiorespiratory physiology in an era of climate change. Canadian Journal of Zoology 87: 835-851.

Kenny, B. J. and K. N. Brown. 2022. ECG T wave. StatPearls Publishing.

Neubauer, P. and K. H. Andersen. 2019. Thermal performance of fish is explained by an interplay between physiology, behavior and ecology. Conservation Physiology. 7(1): coz025.

Vornanen M., A. Badr, and J. Haverinen. 2024. Cardiac arrhythmias in fish induced by natural and anthropogenic changes in environmental conditions. Journal of Experimental Biology. 20:227.

WQCC (Water Quality Control Commission). 2011. Temperature criteria methodology. Policy Statement06-1. Colorado Department of Public Health and Environment.

Zena, L. A., A. Ekström, D. Morgenroth, T. McArley, A. Gräns, M. Axelsson, I. B. Johansen, and E. Sandblom. 2024. Ischemia-induced alterations in the electrocardiogram of salmonid fish. Aquaculture 58: 740482.

Mitigation of Morbidity and Mortality Caused by Iron (Fe) and Iron Oxidizing Bacteria (Archaea) at the Poudre River State Trout Hatchery

Dr. Paula A. Schaffer, Marde Hersh, Dr. Colby L. Wells, Dr. Pete Cadmus

4.1 Introduction

The Poudre River State Trout Hatchery (PRSTH), a Colorado public trout hatchery, has experienced consistent morbidity and mortality of salmonids from hatch to 60 d post swim-up. The well water that feeds PRSTH's rearing units is high in iron, low in dissolved oxygen (DO), slightly acidic, and contaminated by iron-oxidizing archaea (also known as Iron-Oxidizing Bacteria; FeOB). FeOB occupy unique fresh and saltwater niches where ferrous iron (Fe^{2+}) is available and dissolved oxygen (O_2) is limited such as the edges of oxic-anoxic transition zones (Hedrich et al. 2011; Clift et al. 2022). FeOB use electrons released from the oxidation of ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}) to power the electron transport chain (ETC) within their inner membrane (Hedrich et al. 2011). When the Archaea oxidize Fe^{2+} to Fe^{3+} insoluble iron hydroxides precipitate and join with organic waste and biofilm to form a slimy rust colored iron-oxide precipitate complex (IOPC). Rust colored slime can be seen streaming from the opercula and gills of sac-fry and early nektonic age classes. This slime can occlude the branchial cavity and is associated with severe proliferative branchitis (Clift et al. 2022). In affected fish there is severe occlusion of interlamellar spaces by proliferative epithelial cells and there is severe synechiation between adjacent primary filaments. Gram negative prokaryotic colonies mixed with iron-oxide precipitates have been identified histologically in the orobranchial chamber and surrounding gill filaments (Figure 4.1 and 4.2). It is suspected that the oxygen demands of fish produce a low dissolved oxygen microhabitat in the gill chamber that may favor local FeOB proliferation, and small fish size may be most susceptible to entrapment of the IOPC based on function of the orobranchial chamber volume and sieve action of gills and opercula at this age. Managers feel that once fish survive past 60 days post-swim-up they are more resistant to orobranchial chamber occlusion. Rainbow Trout mortality (pick-off) from hatch to 60 d post-swim-up has ranged from 30% to 95%, but other species may be less susceptible. Some hatchery managers and fish biologists propose that species that swim up rapidly into the water column (e.g., Greenback Cutthroat Trout) seem to have improved survival at PRSTH. This is perhaps due to their ability to avoid excessive interaction with IOPC at the bottom of aquaculture tanks at early life stages or perhaps because less sedentary behavior physically flushes IOPC from the gill cavity before occlusion.

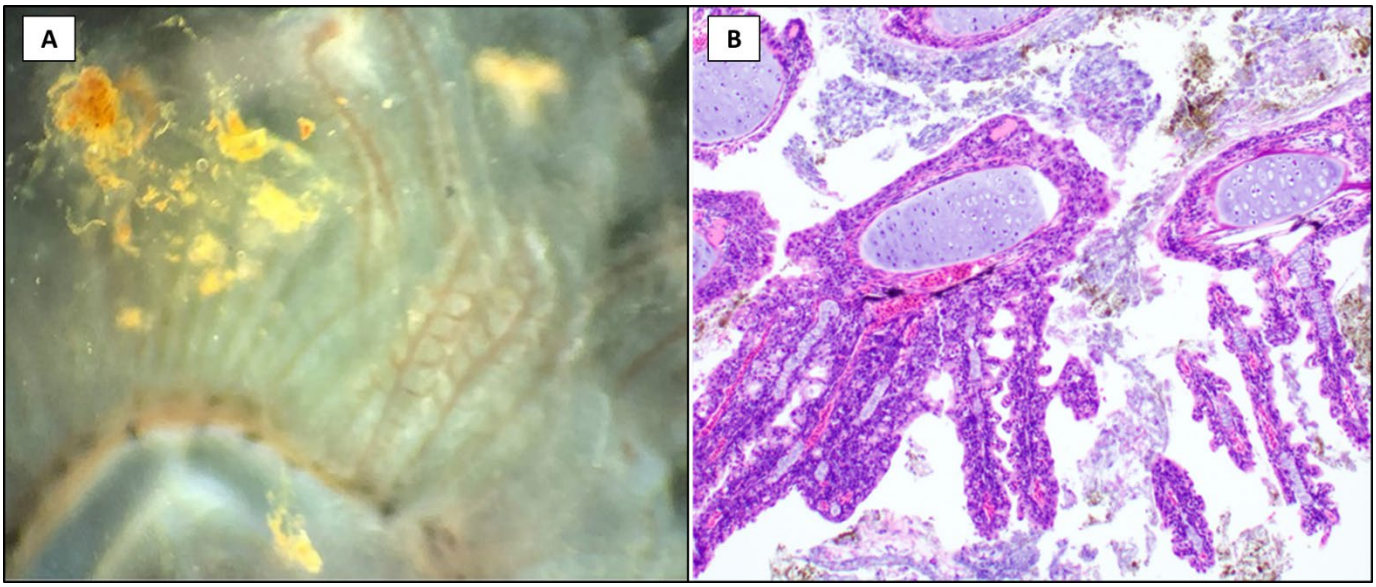


Figure 4.1 (A) Gill cytology from Clift et al. 2022 showing Rainbow Trout from PRSTH. Red-orange iron oxidate precipitate complex is adhered to gill filaments. (B) Histopathology demonstrates that the space between primary gill filaments is occluded by iron-oxide precipitate complex. There is severe branchitis and synechiation between multiple primary lamellae.

We suspect that these Archaea and their impact on the gill are a primary cause of the gradual pick-off that is persistently observed at PRSTH. FeOB have stringent requirements and are not routinely cultured because of these challenges. Previous work identified the FeOB at PRSTH morphologically to the genera *Gallionella* and *Leptothrix* (Clift et al. 2022). Samples have been collected for metagenomics in order to identify the FeOB to species level. Importantly, it is possible that the IOPC could host numerous bacteria and/or that the damage caused by IOPC could predispose to secondary bacterial infections that could compound disease. Metagenomics will help determine if there may be secondary opportunist bacteria that may be contributing to morbidity and mortality.

Even in the absence of FeOB, water high in iron presents a challenge for the survival of aquatic organisms. A suite of potential direct and indirect toxic pathways exist with Fe^{2+} causing mostly direct toxicity as it is most bioavailable and causes oxidative toxic stress while Fe^{3+} causes indirect toxicity as it physically smothers organisms and clogs benthic habitat (Cadmus et al. 2018a,b; Kotalik et al. 2019). Aqueous iron (typically Fe^{2+}) is bioavailable through the gill tissue or the gut. Ferric iron, in the presence of gastrointestinal enzymes can readily be converted to ferrous iron and is absorbed into blood and tissue. Turbidity of ferric iron oxides limits primary production and prevents foraging by trout. As iron oxides (Fe^{3+}) settle, benthic habitat and redds become clogged killing benthic age classes of trout and all autochthonous forage.

Large mortality events were historically observed at PRSTH after power outages when the abrupt stop-start of well pumps would pull iron oxide precipitate and (likely) archaea from well casings. If water is not manually diverted this iron smothers eggs and fry and creates turbid conditions of older age classes. These direct and indirect toxicities are likely in the hatchery setting as well. Iron oxide (rust) and IOPC continually clogs hatchery infrastructure and smothers eggs and sac-fry in rearing tanks.

Intuitively, managers at PRSFH attempted to find mechanical solutions to counteract the direct and indirect stress associated with iron. To prevent the smothering of eggs and sac-fry, staff moved egg and fry rearing to large (1.2 m diameter) round tanks and held eggs and fry in a round perforated (3 mm pore) aluminum sheet suspended ~5cm below the surface. This held eggs near the surface and allowed floc to settle to the 1 m void below the sheet metal. But the inability to remove IOPC led to clogging and bacterial growth (2015-2016). This design was later employed in the form of floating or hanging perforated sheet metal baskets in rearing troughs that allowed cleaning and siphoning below (2021-present). Accumulation of IOPC was avoided by employing Montana Jars (2019) however fungus spread rapidly as daily removal of dead organisms/eggs was not possible. Managers saw the inflamed gill space and "streaming" from opercula as a possible pathogen before the discovery of FeOB. Three 0.5% salt bath treatments every day three days after mortality events (2013-present) had mixed success and have been used regularly since. To kill potential pathogens UV lamps were plumbed upstream of rearing tanks (2013) but were quickly rendered useless as the lens was stained orange and clogged with iron oxides in only days.

Managers worked hard to reduce the frequency and intensity of the pulses of iron oxides delivered when well pumps abruptly stopped and started in a power outage. Managers attempted to remove IOPC buildup in the well casings chemically by injecting chlorine (2013 and 2016). When unsuccessful, the pump was pulled and rehabbed and well screens were mechanically scrubbed (2016 and 2019). These efforts failed to removed FeOB and saw short lived relief from IOPC. Programmable variable frequency drives (VFD) were added to both wells (2020) to slowly turn on wells after a power outage. This reduced the large catastrophic pulses of IOPC when wells abruptly turn on. Head tanks were added upstream of rearing troughs (2018) and were siphoned after power outages. These proved to catch the bulk of IOPC "slugs" as water drained to rearing troughs from middle or upper levels of these head boxes. Managers made efforts to ameliorate poor water chemistry. Degassing columns packed with bio-balls (AKA Packed Columns) were employed above Heath stacks, rearing trough and aquaculture tanks. These are often used to de-gas water coming from deep wells, however at PRSFH these served to passively aerate water with converts some of the ferrous iron to ferric iron. Degassing media (Bio-balls or Koch rings) at PRSTH became completely caked in Fe scale after only a couple weeks of use. Baskets containing sodium bicarbonate (baking soda) were placed in packed columns in an effort to increase pH making Fe precipitate more readily (2017). Sodium bicarbonate needed constant replenishment. Each effort was well matched to the unique issues at PRSTH even when the existence of FeOB was not yet known (<2020) and water chemistry was not yet well characterized. Despite efforts like these survival remained poor.

A series of experiments are underway to examine if mortality of fry could be reduced by management practices that A) converted ferrous iron to ferric iron, B) removed iron oxide precipitates, C) reduced the bioavailability of iron, and D) reduced the presence of FeOBs in the gill space. Additionally we examined possible infrastructure or management practices that might improve survival. Table 4.1 provides a list of management options.

Table 4.1. Management Options

Management Option	Increasing Survival
Completely oxidize ferrous iron to ferric iron upstream of aquaculture operations – this limits survival of FeOB	- $KMnO_4$ continuous infusion with sufficient reaction time upstream of fish culture. - $KMnO_4$ continuous infusion, reaction time, followed by neutralization by a non-toxic sacrificial anti-oxidant such as Sodium Thiosulfate - Continuous infusion of other strong oxidizers and sacrificial buffers as described above. (note: some flocculants might suffice) - Vigorous aeration of water with atmospheric air (Safest Option) - Aeration with pure oxygen from oxygen generators or cylinders, or O_3 if a generator is purchased for WD control. - Increased reaction times
Remove iron oxide precipitates – this limits physical smothering of organisms and reduces contact to IOPC	- Conical tanks or rectangular baffled settling tanks up-stream of aquaculture use - Convert a minority of the >2 hectares (>5 acres) of fishponds to settling ponds. These were retired due to whirling disease - Sand filter arrays - Alternative settling systems - Continuous infusion of flocculants (Note: The IOPC rich water from all but the settling ponds could be diverted to outdoor raceways as the use of this water is not harmful to larger age classes.)
Reduced bioavailability of iron to both fish and FeOB	- Humic acid continuous infusion - Flocculant continuous infusion

Antimicrobial treatments to kill FeOB	- Continuous infusion or pulsed (bath) use of nonspecific antimicrobial treatments
	  - Chloramine T
	  - Formalin
	  - NaCl
	  - $KMnO_4$
	  - Chlorine
	  - Other antibiotic or ectoparasite treatments as identified
	- Continuous infusion or pulsed (bath) use of specific antibiotics in water at optimal dose and contact time
	  - Tetracycline
	  - Enrofloxacin
	  - Others as identified
	- Application of specific antibiotics in feed
	  - Tetracycline
	  - Enrofloxacin
	  - Others as identified

Additional management alternatives	- Promote production of species that exhibit a nektonic behavior as sac-fry (observed in GBNs by PRSTH staff) - Rear species or strains resistant to Archaea (based on life history traits) - Conduct hard on-off well cycles weekly while water is diverted from aquaculture uses - Install programmable solenoid (abrupt) or geared (gradual) valves, immediately downstream of wells, that divert water from aquaculture uses in the three to five minutes after a well start up. - Employ tank designs, aeration, and flows that increase DO and prevent settling of IOPC.

In Phase 1 (Spring 2024) we compared historical rearing practices (control) to intense aeration upstream of rearing troughs, and to use of a novel miniaturized modular water conditioning platform (MMWCP) that completely oxygenated water, added reaction time, and then removed some iron oxides from water (Figure 4.3). In Phase 2 (Fall 2024), the MMWCP and infrastructure from Phase 1 was utilized but at markedly reduced flow rates, which further increased the amount of iron-oxides removed by the MMWCP and further increased reaction time. Phase 3 (Fall 2024) compared pulsed weekly treatments of nonspecific antimicrobial agents common in hatchery settings for removal of ectoparasites and bacteria (Chloramine T, formalin, $KMnO_4$, and NaCl).

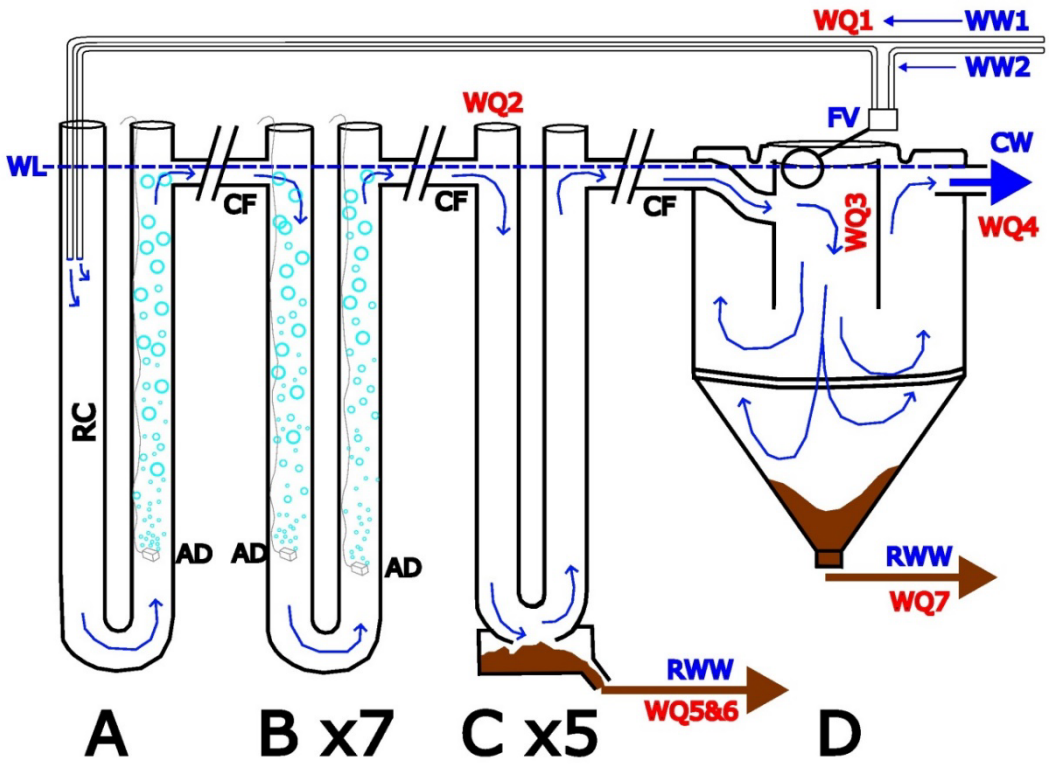


Figure 4.3 Modular Miniaturized Water Conditioning Platform (MMWCP) – The MMWCP is an aquaculture water conditioning platform that lets PRSTH test the needed intensity of common water conditioning techniques. Aeration (air stones), degassing packed columns, and reaction time are used to ensure ferrous iron is converted to ferric iron. It consists of a series of 4" NPS PVC columns (A, B and C; 10.1 cm diameter x 207-230 cm height) and a conical tank (D; 320 L; INFD85-30, Den Hartog Industries, Hospers, Iowa, USA). A calibrated flow of well water enters the system at WW1. Additional well water (WW2) flows are restricted by a float valve (FV) calibrated to the water line (WL; 207cm height) that represents the hydraulic head of the system. This float valve only engages when WW1 experiences a clogged valve (common) or extra flow is temporarily needed downstream of the MMWCP. Each of the 14 vertical columns (A and B) or ten vertical sumped columns (C) can be used as a resting chamber (RC) and/or as a column receiving diffused O2, O3, or pressurized atmospheric air (AD). Sumps in U-shaped columns (C) and a conical tank (D) were employed to determine the reaction time needed to convert ferrous iron to iron oxides (RWW. None observed in Phase 1). Conditioned water is then delivered to aquaculture systems during experiments. Quick connect cam-fittings (CF) were used throughout to allow addition of columns and settling systems or conversion from series to parallel orientation. CF also allowed the miniaturized system to be dismantled and reassembled indoors. Water quality assessments at WQ1 through WQ7 were sampled regularly and flows assessed to inform design or scope of work for permanent water condition systems in the future.

4.2 Methods

4.2.1 *Metagenomics*

Two ~200 microliter samples of floc (IOPC) were siphoned from the base of the head tank (fishless) and rearing troughs by Pasteur pipette. IOPC was carefully dissected from the branchial chambers of seven fish with grossly evident orange branchial occlusions. The samples were stored in 100% ethanol and frozen at -80ºC until funding for evaluation is secured.

4.2.2 *Preliminary Histopathology*

A subset of fish with grossly evident orange material in the orobranchial cavity were euthanized with buffered MS-222 and immersion fixed in 10% neutral buffered formalin for greater than 24 hours. The fish were routinely processed and embedded dorsal side down in paraffin blocks. Blocks were trimmed until the level of the orobranchial cavities was reached, then they were sectioned at seven microns. Sections were mounted on charged slides and stained routinely with haematoxylin and eosin for evaluation by a diplomat of the American College of Veterinary Pathologists.

4.2.3 *Aquaculture*

Hatchery rearing units in the State of Colorado obtain eggs spawned from wild populations and private or public sector hatcheries from across North America. Poudre Hatchery Rearing Units see similar mortality regardless of species. Species were selected to match seasonally available eggs. Eyed eggs of Hofer-Harrison Rainbow Trout females x Gunnison River Rainbow males (HXG) from Gunnison State Hatchery were used in Phase 1 (early spring of 2024) and Rainbow Trout (*Oncorhynchus mykiss*) from Troutlodge (Phase 2 and 3) were enumerated and assigned to aluminum mesh (3.0 mm pore size) egg baskets in 26 x 36 x 122 cm aluminum rearing troughs (Figure 4.4A) with a standpipe maintaining a depth of 22 cm (96.6 L). Troughs received flow-through water from high Fe^{2+} wells at 7.8 L/min in Phase 1 with a minimum reaction time of seventeen minutes (maximum reaction time of 51 minutes) and 757 mL/min in Phase 2 with a minimum reaction time of 2hrs 34 min. A handheld flow meter (aka: rotometer or floating weight flow meter) attached to a carpenter's string level was used to correct flows for troughs daily to be within 13% of target. Mesh baskets were removed from an experimental unit after 60 d post hatch.

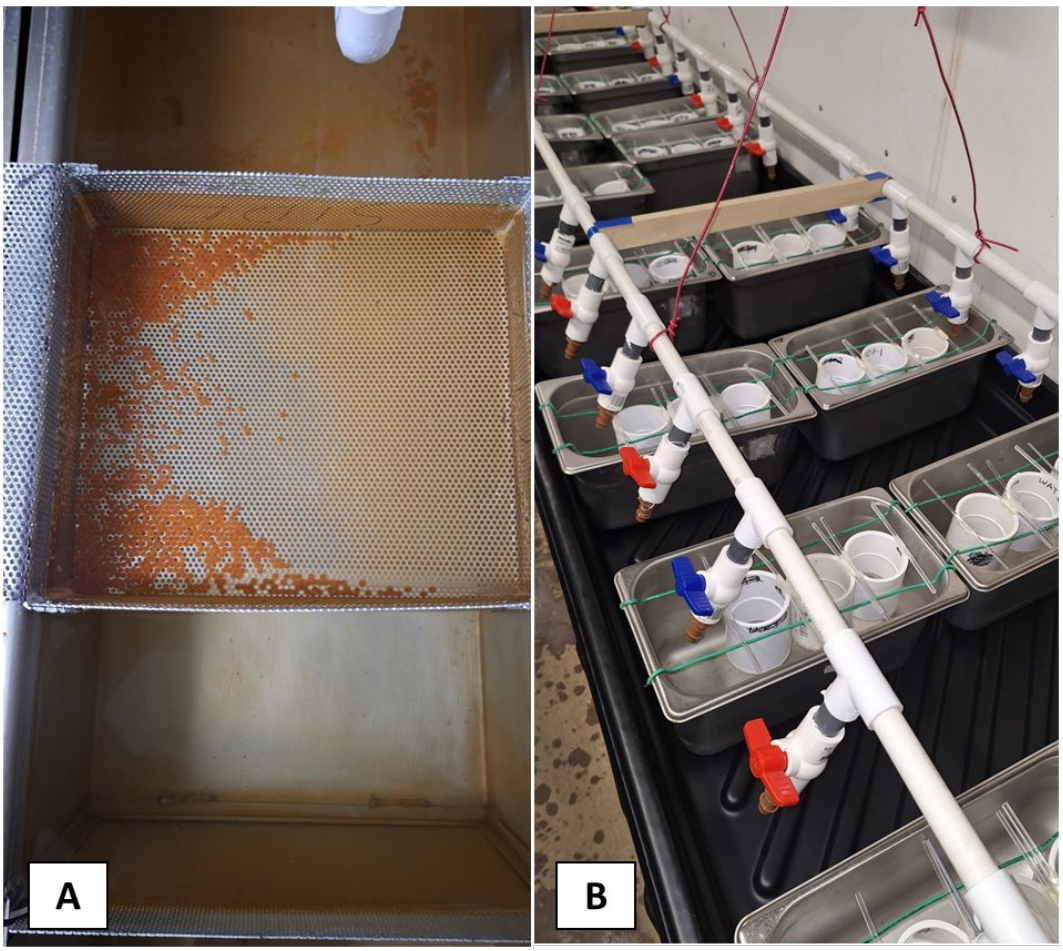


Figure 4.4 A) Perforated baskets suspended in rearing troughs. Pores retain eggs and sac-fry while allowing IOPC to settle out. B) Stainless steel aquaria (Phase 3) were used to investigate non-specific antimicrobial treatments. Shown here with small removable egg incubation cups.

Experimental units in Phase 3 consisted of 15 x 30 x 15cm (5.56 L) stainless steel aquaria (Polar Ware 5.56 L Hotel Pans. Model G12066, Sheboygan, WI, USA). Stainless aquaria received 378 mL/min of oxygenated water from an aerated head tank (Figure 4.4B). Flows were calibrated daily using a handheld flow meter. Hotel pans were calibrated within 25% of target flow rate daily as previously described. Pans had a mesh lined hole in the wall to maintain a depth of ten cm (2,225 mL). All pans were held in water baths to ensure the same temperature in all experimental units. Eggs were placed in small mesh lined egg cups (Figure 4.4B) and transferred to hotel pans after hatch.

For all experiments, feeding was delayed until 100% of individuals in an experimental unit showed complete ventral fusion. This was observed May 6th, 2024 for Phase 1, September 20th for PR2E in Phase 2, and September 25th for remainder of Phase 2 and Phase 3. Feeding in PR2E, the treatment receiving water from the WTP, was started five days before PR2W and PR1, likely due to improved water chemistry and temperatures. Fry were fed ad lib hourly, for eight hours a day per day per standard hatchery practice (BioVita Starter #0 Crum, Bio-Oregon Life Stage Diets for Fish, Westbrook, ME). All troughs and hotel pans were scrubbed of biofilm

and cleaned of precipitate and waste daily. Mortalities were enumerated for each experimental unit daily.

4.3 Experimental Methods

4.3.1 *Phase 1*

Historical aquaculture infrastructure employed a degassing column (19 L) of Bio-Balls (Pentair, Apopka, FL, USA) into a head tank with minimal aeration. Four troughs (n=4) representing this water conditioning served as the control for Phase 1 and Phase 2.

Conversion of Fe^{2+} to Fe^{3+} was performed using extreme aeration by large regenerative blowers (Sweetwater SST15, Lake Forest, IL, USA). Complete saturation (100% DO) was obtained in head tanks feeding non-control troughs. This treatment (Aeration, n=4) was considered an inexpensive improvement requiring no new infrastructure beyond modern blowers. Atmospheric O_2 is a mild oxidizer and can convert Fe^{2+} to Fe^{3+}. The remote location of Poudre hatchery made delivery and storage of other oxidizers (example: $KMnO_4$ or bottled oxygen) difficult or a health concern.

Complete conversion of Fe^{2+} to Fe^{3+} and the precipitation of iron oxides requires a long reaction time and an oxidizer. A custom modular miniaturized water conditioning platform was constructed with an array of fourteen aerated columns (10.16 cm diameter x 227 cm water column height), ten resting columns with sumps, and one large conical tank. Mortality, survival, growth and assimilation efficiency of this treatment level (Treatment, n=4) was compared to Aeration and Control by REGWQ multiple comparison within ANOVA.

4.3.2 *Phase 2*

Phase 1 was repeated using reduced flows (400 mL/min per experimental unit). This allowed better approximation of the Fe removal possible in industrial sized treatment plants. The reduced flows reduced availability of ferrous iron to FeOB, reduced direct toxicity of ferrous iron to the gill surface, and reduced ferric IOPC.

4.3.3 *Phase 3*

Archaea are a distinct domain of single-celled microorganisms that are similar to bacteria but that differ significantly in their genetics, biochemistry, and evolutionary history. We hypothesized that the FeOB at PRTSH may be sensitive to nonspecific antimicrobial agents utilized in a hatchery setting. For this reason, we elected to investigate whether agents like Chloramine T (an oxidizing agent, Halamid Aqua, Syndel, Ferndale, WA, USA), formalin (as an alkylating agent, Parasite-S 37%, Syndel, Ferndale, WA, USA), and potassium permanganate ($KMnO_4$, an oxidizing agent), could effectively reduce Archaea load and thereby reduce the production of IOPC. We also chose to try sodium chloride bath treatments as a nonspecific measure of supportive care (American Stockman Solar Mixing Salt Kiln Dried; Compass Minerals, Overland Park, KS, USA). Treatment regimens are listed in Table 4.2.

Table 4.3 Antimicrobial treatment regimens applied weekly at PRU

ChloramineT	12 ppm x 60 min
ChloramineT	200 ppm x 60 min
Formalin	40 ppm x 60 min
Formalin	80 ppm x 60 min
$KMnO_4$	10 ppm x 30 min
$KMnO_4$	100 ppm x 5 min
Sodium Chloride	0.5% x 30 min
Sodium Chloride	1.5% x 15 min

4.3.4 *Water Chemistry*

Weekly (+/- 2 d) dissolved (0.45 µm Nylon filter) and total (unfiltered) iron were sampled in 15 mL polypropylene falcon conical bottom tubes for analysis by ICP-MS. Each experimental unit was sampled from a statistical block. This ensured one observation from each type of treatment and control(s) weekly and represented each block fairly over the duration of the experiment. Each week, metal samples (dissolved and total), and a ferrous iron assessment (optical Hach colorimetric kit DR890) was taken from the head tank (aka, head box) of each of the three treatments. Ferrous iron observations were recorded on the data sheets containing DO and temperature.

Daily dissolved oxygen and temperature was assessed with a YSI ODO (optical dissolved O_2, YSI Incorporated, Yellow Springs, Ohio, USA) that was calibrated daily. All experimental units (4 troughs) on each treatment type (Control, Aerated, MMWCP) were assessed and recorded on sheets daily. Head boxes were also assessed daily.

In Phase 3, the total and dissolved iron was sampled similarly, and the statistical blocks were sampled systematically each week. DO and temperature were sampled daily as previously described.

A ThermoScientific iCAP 6000 ICP-OES was used to analyze for Fe (ferrous + ferric), Ca, Mg, K, Na, Cu, Zn, and Mn. Samples and solutions were preserved with ultra-pure (Ultrex®II, J.T. Baker or equivalent) nitric acid (1 mL per L or one drop per 5 mL of sample). Matrix solution for blanks standards and dilutions were made of deionized water (Barnstead Nanopure system; Thermo Fisher Scientific). Five-point calibration for each element was conducted prior to each batch of twenty samples and was analyzed after each batch to ensure no drift. At a minimum the following quality assurance samples (QA) were analyzed. Each batch was accompanied with at least one duplicate sample at the time of collection and at least one sample split just prior to analysis, each flagged if duplicate or split was >5 or 10% from original. Blanks were flagged if greater than 5% of detection limit suggested by manufacturer or detection limit calculated from previous batches. External quality assurance standards for each element were assessed every ten samples and were flagged if >5% from nominal or more frequently at the analyst's discretion. External standards obtained from nationally certified firms were NIST -Traceable to the SRM 3100 Series. Standards had a certificate of analysis and SDS that guaranteed accuracy (99.999% certified accuracy to ±0.3%) and stability. An yttrium internal calibration standard was continuously introduced into the plasma along with each sample. If any QA/QC flags were

observed instrument was recalibrated and all samples of that batch were reanalyzed. Samples found above the highest standard in the calibration curve were diluted (1:2, 1:5 or 1:10) and reanalyzed in a subsequent batch. Ferrous Fe was assessed immediately after collecting unpreserved samples using a phenanthroline colorimetric reagent in a Hach DR/850 colorimeter (Hach, Loveland, Colorado, USA. Method 8146. DL=0.02 mg/L). No shelf stable ferrous iron standard was available; however, the absorbance of the instrument was validated daily.

Valves of every experimental unit in every block of Phase 3 were opened daily to flush iron oxide precipitates. Hotel pans were removed from the flow of water; the valve was opened completely, then shut.

4.3.5 *Histopathology*

Due to potential fungus loads, 30 d post swim-up fish were sampled for histopathology on Oct 18th, 2024 (Phase 2). Phase 1 and Phase 2 were sampled at the completion of the experiment (near 60 d post swim up). Histopathological assessment of disease, occlusion of gills space, fungal infection, and iron accumulation in tissue was performed by the Colorado State University (CSU) Veterinary Diagnostic Laboratory. Three fry were sampled from each experimental unit in Phase 2 on Oct 21st. Two fry were sampled from each experimental unit in Phase 3 on Oct 21st. Fish were preserved in 10% neutral buffered formalin. When there was 100% mortality in a hotel pan, two fish were sampled from a different block in the same treatment (randomly decided by coin toss). Similarly, if one trough of a treatment was completely void of fish due to mortality, three fish were sampled from a different block of the same treatment (randomly decided by coin tosses or random number generator).

4.4 Results

4.4.1 *Survival of Phase 1*

Statistically significant improvement ($F_{2,9}$=8.09, p=0.009) in survival from hatch to 60 d post swim up was observed in the treatment using the MMWCP (mean= 0.330) when compared to the control (mean=0.069, p=0.01;) or the use of heavy aeration in the head tank (mean=0.132 p=0.02; Figure 4.5). Aeration of the head tank offered minimal improvement relative to the control but not significantly significant (p=0.38). The same stain of trout, Hofer-Harrison x Gunnison River Rainbows (HXG), experienced 81%, 97% and 99% mortality at PRSTH in March 2023, June 2022, and February 2022, respectively.

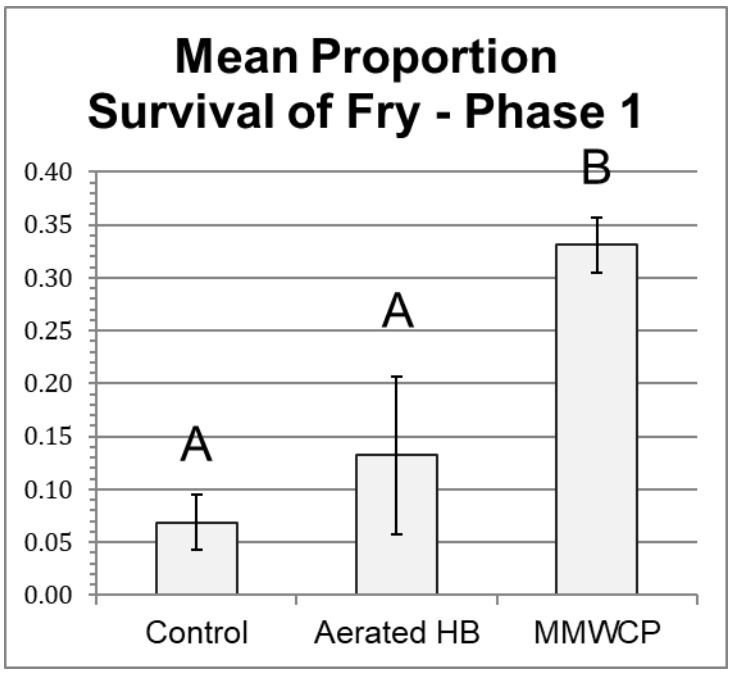


Figure 4.5 Proportion survival of HXG from hatch to 60 d post swim-up. Control water source represents well water that only passes over a degassing "packed column." Aerated HB represents a treatment that improved on the control infrastructure of the control by adding vigorous aeration in the head box upstream of rearing troughs. MMWCP expands on the Aerated HB water conditioning by adding 17-54 min of vigorous aeration and time for reaction, perhaps adding minimal removal of iron oxides. Statistically significant differences between groups is denoted by different letters A B using REGWQ comparison in ANOVA.

The flows passing through the MMWCP during Phase 1 simulated what was historically used in rearing units at PRSTH. These flows prevented settling of iron oxides in the sumps of C and D in Figure 4.3. This was confirmed by total iron concentrations at WQ5, WQ6 and WQ7, which were only elevated relative to WQ3 and WQ4 after power outages. Qualitatively, deposition of IOPC was observed to be substantial in all replicates of all three treatments of Phase 1. Increased survival was likely attributable to the high oxygen saturation and the conversion of Fe^{2+} to Fe^{3+}.

4.4.2 *Water Chemistry*

Iron concentrations from the well (measured at PR2) fluctuate from <0.005 mg/L to 2.5 mg/L when water is not noticeably turbid. During pulses of heavy particulate iron oxides Fe concentrations are significantly higher. Ferrous iron was not observable in the head tanks receiving simple aeration from a blower, nor was it observed downstream of the MMWCP. Ferrous Iron was present (0.01 mg/L to 0.045 mg/L) in head boxes representing historical use of only degassing columns. But typically these values were near the detection limit of 0.020 mg/L and below. It is likely FeOB can proliferate at concentrations well below this level.

In Phase 1 studies no significant difference in total iron or dissolved iron or a dissolved/total ratio was detected between the three treatment types. Phase 2 is pending but will better remove Fe precipitates and inform if physical smothering of fry by iron oxides is a source of pick off. Chemistry from Phase 2 and 3 is ongoing. Weekly assessment of MMWCP performance is ongoing.

4.4.3 *Preliminary Histopathology*

Large prokaryotic colonies aggregated with orange IOPC were present in the orobranchial chambers and prokaryotic colonies and were also noted in the recesses of the nares (Figure 4.6). Small fragments of feed material were entrapped within the orobranchial chambers, often closely associated or intermixed with prokaryotic colonies and IOP. The interlamellar spaces were mildly to moderately occluded by proliferative branchial epithelium.

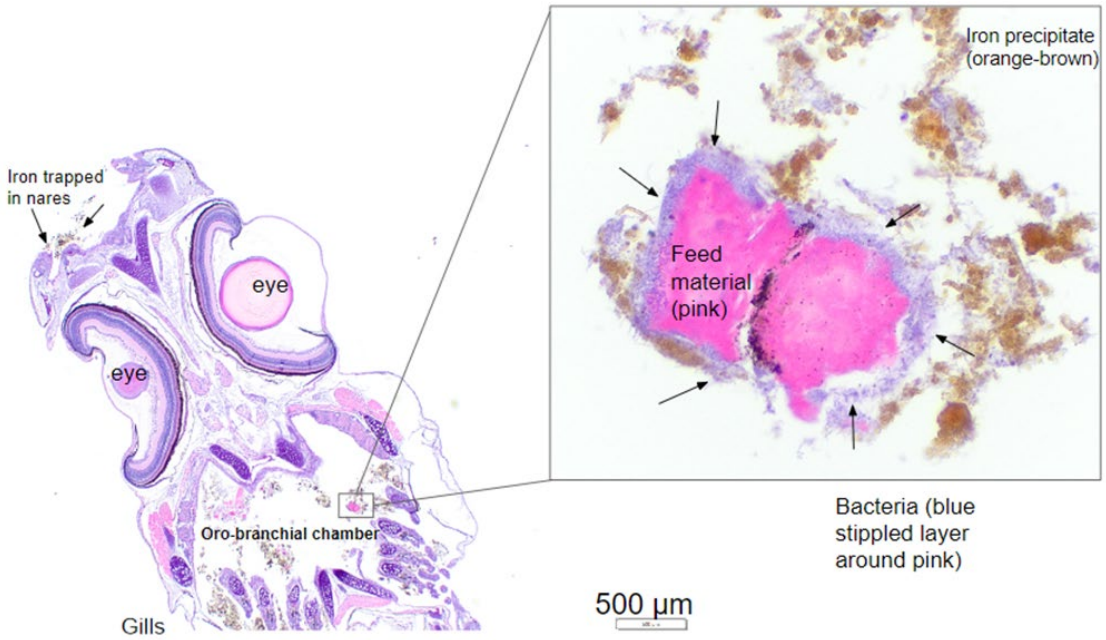


Figure 4.6 Heavy burden of IOPC, food waste, and prokaryotic communities observed in the orobranchial cavity and nares of a "near-morbid" fry during Phase 1 experiments (30 July 2024).

4.4.4 *Metagenomics*

Samples for metagenomics are preserved and archived. Analysis will be performed if funding is secured. We expect this to be a fruitful endeavor that will clarify the microbiome of IOPC.

4.5 Discussion

With no intervention, standard practices using direct well water at the PRSTH resulted in cultivation of fish in substantially high total and dissolved iron concentrations and proliferations of FeOB that smothered eggs and fry and which impinged respiration of nektonic life stages by occluding the orobranchial chambers and inducing severe branchitis. This has resulted in historically documented high morbidity and mortality rates. With the introduction of a custom water conditioning system (MMWCP) at high flow rates there was substantial improvement in survival, likely due to the complete conversion of Fe^{2+} to Fe^{3+}(bad for FeOB) as a result of sustained and thorough saturation of water with dissolved oxygen (good for fish). At the high flows of Phase 1, little removal of Fe was achieved. Phase 2 will be run at flows which should let Fe settle out in columns C and tank D of Figure 4.3. Unfortunately, preliminary results from

Phase 2 suggest large fungal loads on fry and surfaces of rearing troughs. Fungus load and mortality is especially large downstream of the MMWCP, which produces warmer water. Alternative configurations (parallel settling tanks) might increase MMWCP output while maintain slower flows. Treatment trials with Chloramine T, $KMnO_4$, formalin, and NaCl are ongoing. Results from this study could be used in concert with the various configurations of the modular water conditioning platform.

A subset of fish were evaluated by light microscopy and demonstrated novel changes not reported previously by Clift et al. (2022). For instance, samples showed entrapment of feed material in the orobranchial chamber (Figure 4.1, 2 and 6). Feed material entrapped in the orobranchial chambers may exacerbate the growth of FeOB by acting as a nutrient source or may be a permissive factor in the development of secondary bacterial infections of the gill, which could compound the FeOB associated pathology and decrease survival. This histologic finding emphasizes the importance of the pending metagenomics project. Histopathology cannot distinguish archaea or bacteria to species level, and it is possible that mixed populations are present. Clift et al. (2022) indicated at least two FeOB species present based on morphologic identification. At that time diagnostic work was focused on the FeOB rather than bacteria. A positive observation of *Pseudomonas sp.* was observed prior to 2020. If multiple species of FeOB are present or if there are both FeOB and non-archaea present it is possible that they have varied susceptibility to antimicrobial treatments. If non-archaea are identified and are suspected to be pathologic, specific antimicrobials approved for bacteria in aquatic environments such as tetracycline or enrofloxacin could be considered either as periodic treatments or as constant drips. Metagenomic characterization of the IOPC microbiome might prove helpful in guiding future treatment trials. Histopathology analysis comparing three treatment groups in Phase 1 is pending.

Histologic samples processed in this study were sectioned in the frontal plane, which offered a new perspective than the midsagittal sections evaluated by Clift et al. (2022). The frontal plane sections (Figure 4.6) showed IOPC lodged in the nares of fish in addition to within the orobranchial chambers as previously reported. Orobranchial occlusion by IOPC could interfere with successful prehension and deglutition of feed, and could be a factor in ill thrift at PRSTH. Additional work is needed to determine if impaction of the nares by IOPC could interfere with foraging behavior. If so, this factor could also contribute to poor weight gain and poor feed conversion in addition to the high energetic cost of branchitis.

4.6 References

Cadmus, P., S. F. Brinkman, and M. K. May. 2018. Chronic toxicity of ferric iron for North American aquatic organisms: Derivation of a chronic water quality criterion using single species and mesocosm data. Archives of Environmental Contamination and Toxicology 74(4): 605-615.

Cadmus, P., H. Guasch, A. T. Herdrich, B. Bonet, G. Urrea, and W. H. Clements. 2018. Structural and functional responses of periphyton and macroinvertebrate communities to ferric Fe, Cu, and Zn in stream mesocosms. Environmental Toxicology and Chemistry 37(5): 1320-1329.

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.

Hedrich, S., M. Schlömann, and D. B. Johnson. 2011. The iron-oxidizing proteobacteria. *Microbiology (Reading, England)* 157(Pt 6): 1551-1564.

Kotalik, C. J., P. Cadmus, and W. H. Clements. 2019. Indirect effects of iron oxide on stream benthic communities: Capturing ecological complexity with controlled mesocosm experiments. Environmental Science and Technology 53(19): 11532-11540.

Field based temperature standards for Bluehead Sucker (*Pantosteus discobolus)*, Flannelmouth Sucker (*Catostomus latipinnis*), and Roundtail Chub (*Gila robusta)*

Darian Woller (CSU Master's Student), Dr. Tawni B. R. Firestone

5.1 Introduction

Water temperature affects many biological processes among aquatic ectotherms such as growth and reproduction (Killen 2014; Hasnain et al. 2018; Volkoff and Rønnestad 2020). When temperatures exceed the optimal range fish can tolerate, they experience thermal stress which may affect growth, increase metabolic demands, induce immobilization, and cause possible mortality (Fry 1947; Cech and Moyle 2004; Donaldson et al. 2008; Handeland et al. 2008). Identifying optimal temperature ranges for aquatic organisms is a valuable tool for informing habitat restoration, understanding population dynamics, and developing risk assessments for vulnerable populations (De Vries et al. 2008; Todd et al. 2008; Dibble et al. 2021; Li et al. 2022). Unfortunately, few studies have focused on identifying temperature ranges for threatened species or species of conservation concern (Coutant 1977; Beitinger et al. 2000; EPRI 2011; Jonsson 2023). In Colorado, the Colorado Department of Public Health and Environment (CDPHE) lists at least 25 species with no temperature data with 19 of those being species of conservational interest.

Development of protective temperature criteria for fish have focused on laboratory assessments of acute and chronic responses (Todd et al. 2008). The Colorado Water Quality Control Commission (WQCC) recommends the use of two acute temperature tests when developing temperature standards to protect aquatic life: incipient lethal temperature tests (ILT) and critical thermal tests (CTM) and are performed in a controlled laboratory setting. Each experimental approach aims to define optimal temperature ranges based on a set acclimation temperature. However, it is important to acknowledge that each test evaluates physiological responses to water temperature differently. Fish used in ILT tests are transferred from an acclimation temperature to a constant higher or lower temperature. ILT tests are used to evaluate at which temperature 50% of the fish experience mortality (Fry 1947). Fish used in CTM trials start at an acclimation temperature and the water is constantly increased (CTMax) or decreased (CTMin) until the fish loses its ability to remain in an upright position (loss of equilibrium (LOE); Currie et al. 1998). Both laboratory tests are similar in respect to pre-trial acclimation. Acclimation temperature is defined by the WQCC (WQCC Policy 06-1) as the temperature selected within a fish's tolerance zone at which the fish is held prior to temperature experimentation, usually 14-30 days (Becker and Genoway 1979; Bennett and Beitinger 1997). Together, ILT and CTM data can be used to estimate the optimal temperature ranges for fish species to inform management and to develop protective temperature criteria (Brungs and Jones 1977; EPA 1986; Todd et al. 2008).

Over the past few decades, a growing number of studies have investigated whether laboratory derived temperature standards are representative of what fish in wild populations can tolerate (Konecki et al. 1995; Rodnick et al. 2004; Wehrly et al. 2007; Schofield et al. 2009; Payne et al.

2016; Chen et al. 2018). Two fundamental limitations for ILT and CTM trials and their extrapolation towards fish under natural temperature regimes is the use of acclimation temperature and not accounting for stressors that fish experience in their natural environment. Acclimation temperatures are used to define the relative temperature history and exposure of the fish and is typically viewed as a stable physiological state at which comparisons can be made among temperature treatments and fish species (Bates and Morely 2020). However, temperatures in the environment rarely remain at a constant state and vary both on the diel and seasonal scale (Wehrly et al. 2007). Therefore, the acclimation temperature that fish are held in for weeks prior to laboratory temperature tests does not reflect the fluctuating temperatures wild populations are exposed to and is absent of other abiotic and biotic stressors (Lutterschmidt and Hutchison 1997; Mandeville et al. 2019). Thus, laboratory temperature tests may be incorrectly defining temperature standards. Field-based temperature tests may yield different results than those conducted in a laboratory setting because fish that are collected in the field are exposed to natural temperature fluctuations, dissolved oxygen fluctuations, changes in water flow, and differences in other water quality factors (Desforges et al. 2023). To combine both laboratory temperature tests and natural fluctuations of temperature and water quality parameters, field-based temperature studies should be considered in which laboratory tests are conducted in the field with field caught fish and water used directly from where the fish were collected. To ensure that laboratory derived ILT and CTM results protect wild fish populations, field-based studies that use similar methodologies are needed to determine if results from the laboratory are different from field results.

The Bluehead Sucker (*Pantosteus discobolus*), Flannelmouth Sucker (*Catostomus latipinnis*), and Roundtail Chub (*Gila robusta:* herein after listed as the three species) are species of conservation importance in western Colorado. The three species were historically distributed in the Colorado River, Weber River, Bear River, and Snake River drainages and currently occupy less than 50% of this range (Bezzerides and Bestgen 2002). The Colorado River Fish and Wildlife Council (2004) initiated an agreement with multiple agencies to implement conservation measures in states that the three species occupy. Current research has indicated that hybridization, habitat fragmentation, and fluctuating water temperatures are main predictors of population declines (Bezzerides and Bestgen 2002; Compton et al. 2008; Jones and Petreman 2013).

In lotic systems, larvae are highly vulnerable to environmental stressors, largely from lacking the ability to move from undesirable habitats due to their limited swimming ability and tendency to passively drift (Dudley and Platania 2007; Souchon and Tissot 2012; Dahlke et al. 2020). Streams in the western United States are intensely managed for water use and decreasing average annual streamflows have been observed (Reynolds et al. 2015). Dams have also impacted the temperature of western streams, where reservoir withdrawals, habitat fragmentation, and altered flows have resulted in unnatural increases and decreases in water temperatures (Bezzerides and Bestgen 2002; Dibble et al. 2021). Droughts are also predicted to become more frequent, leading to overall increased water temperatures and reduced water availability (Udall and Overpeck 2017). Under these conditions, larval fish may be subjected to extreme temperature conditions affecting growth and survival (Keller and Klein-Macphee 2000; Jeffries et al. 2016). As these abiotic stressors continue to disturb aquatic systems, defining temperature ranges for the three species is needed.

The purpose of our study is to determine if temperature tests should be conducted in a controlled laboratory setting or in the field with field-caught larval fish and stream water. We conducted field-based temperature tests using standard laboratory practices (ILT and CTM) alongside Roubideau Creek, a tributary of the Gunnison River located near Delta, CO. All larval fish were collected within 24 hours of the temperature trials from Roubideau Creek. We conducted our first field season and are currently comparing our results to previously published laboratory studies to determine if temperature standards should be developed in the laboratory or the field. We hypothesize that field derived temperature tolerance data will show higher upper temperature tolerance ranges and reduced lower temperature tolerance ranges compared to laboratory tests. By addressing field versus laboratory derived temperature standards, our study aims to provide valuable information and assist in the conservation towards the Bluehead Sucker, Flannelmouth Sucker, and Roundtail Chub.

5.2 Methods

5.2.1 *Study Area*

Roubideau Creek is an intermittent tributary of the Gunnison River located near Delta, CO. Our study site was located at the confluence of Roubideau Creek and Potter Creek (Figure 5.1). Annual hydrologic inputs of the Gunnison River and its tributaries comprise of spring runoff from snowmelt throughout the months of April to July (Regonda et al. 2006). HOBO temperature and conductivity loggers were placed near our site in Roubideau Creek to collect yearly temperature data, with readings collected every four hours.

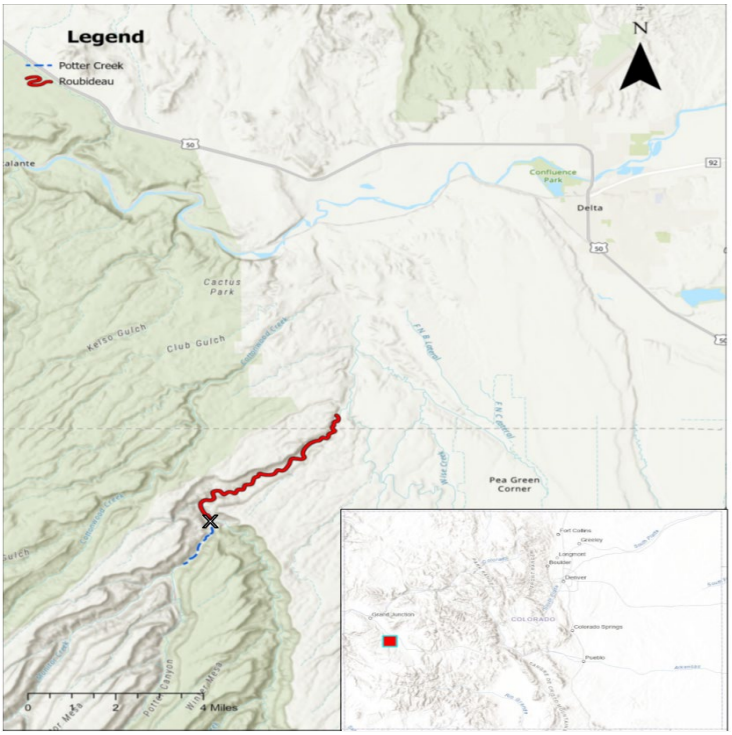


Figure 5.1 Location of study site for three species temperature tests near Delta, Colorado 2024. Tributaries are denoted ↓ Roubideau Creek and - - - Potter Creek. "X" marks approximate location of where mobile lab was stationed. Bottom Right: Inset Location of Study Site; study location.

5.2.2 *Field Collection and Larval Holding*

To detect when larval presence began in Roubideau Creek, three species spawning surveys were conducted weekly using driftnets to identify hatched catostomid eggs. We noted adult three species spawning at the beginning of May, thus drift net sampling efforts began in mid-May with larvae first being detected on May 23rd.

Larval fish sampling efforts occurred from May 26th-June 2nd and June 3rd -June 10th. Sampling occurred in the early morning with the use of dip nets (47 x 28 cm net opening; 38 cm in depth) and were concentrated in low-velocity, bankside habitat. Once collected, larval fish were placed into five-gallon buckets and transferred into a holding tank with stream water and aeration. The holding tank reflected the stream's diel temperature fluctuations as tank water was refreshed with stream water. All larvae were held for one day prior to trials to reduce stress from capture and handling. Since larval identification is difficult with live fish, larvae identification was conducted after temperature trials were completed. Larvae were fed 4 mL of *Artemia salina* daily throughout the holding period to ensure that survival was not influenced by starvation. *Artemia salina* were hatched in a conical tube with 1 g L^{-1} of 25 ppt aerated salt water and incubated for 24 hours at approximately 26ºC.

5.2.3 *Mobile Laboratory Setup*

A mobile laboratory was used to conduct streamside upper ILT (UILT), lower ILT (LILT), CTMax, and CTMin for the three species larvae. The laboratory setup was constructed within a 16 ft enclosed trailer that was modified to achieve multiple ILT treatment temperatures. The mobile laboratory was stationed approximately 40 feet from Roubideau Creek, next to the confluence of Potter Creek (Figure 5.1). Electricity for the tank and trailer system were supplied by two gas generators grounded by copper rods.

Stream water was pumped from Roubideau Creek using a submersible pump (35 ft head pressure at 60 gallons min^{-1}) and filtered through a 100 µm stainless mesh screen filter into two 106-gallon insulated storage tanks located outside of the trailer and covered from the elements. The cold storage tank was regulated by an inline chiller (Ice Pod Chiller ®) and immersion chiller (Frigid Units) set to 4°C and the hot storage tank was regulated by a series of submersible heaters (Process Technology)set to 40°C. Water from the hot and cold storage tanks were then pumped into the trailer's head tanks using submersible utility pumps (25 ft head pressure at 28 gallons min^{-1}) located in each tank.

The experimental ILT tanks contained in the mobile laboratory consisted of two racks. Each rack held five rows of ten, 2-L tanks, resulting in 50 tanks per rack (Figure 5.2). One rack was designated for LILT treatment temperatures and the other UILT treatment temperatures. Each row was supplied by a head tank that was set to a defined temperature. The head tanks were regulated by mixing hot and cold water from the two storage tanks using a temperature controller (B-series Love Controls Division) and solenoid valves. Each storage tank received diffused air from a blower system that was submerged in the outside storage tanks. Dissolved oxygen (DO), flow, temperature, and pH of the water in the tanks were monitored throughout the experiments with a YSI ProODO and YSI pH instrument. Effluent water from the experimental tanks were recirculated back to the storage tanks to reduce sedimentation from stream water and reduce the energy needed for cooling and heating.

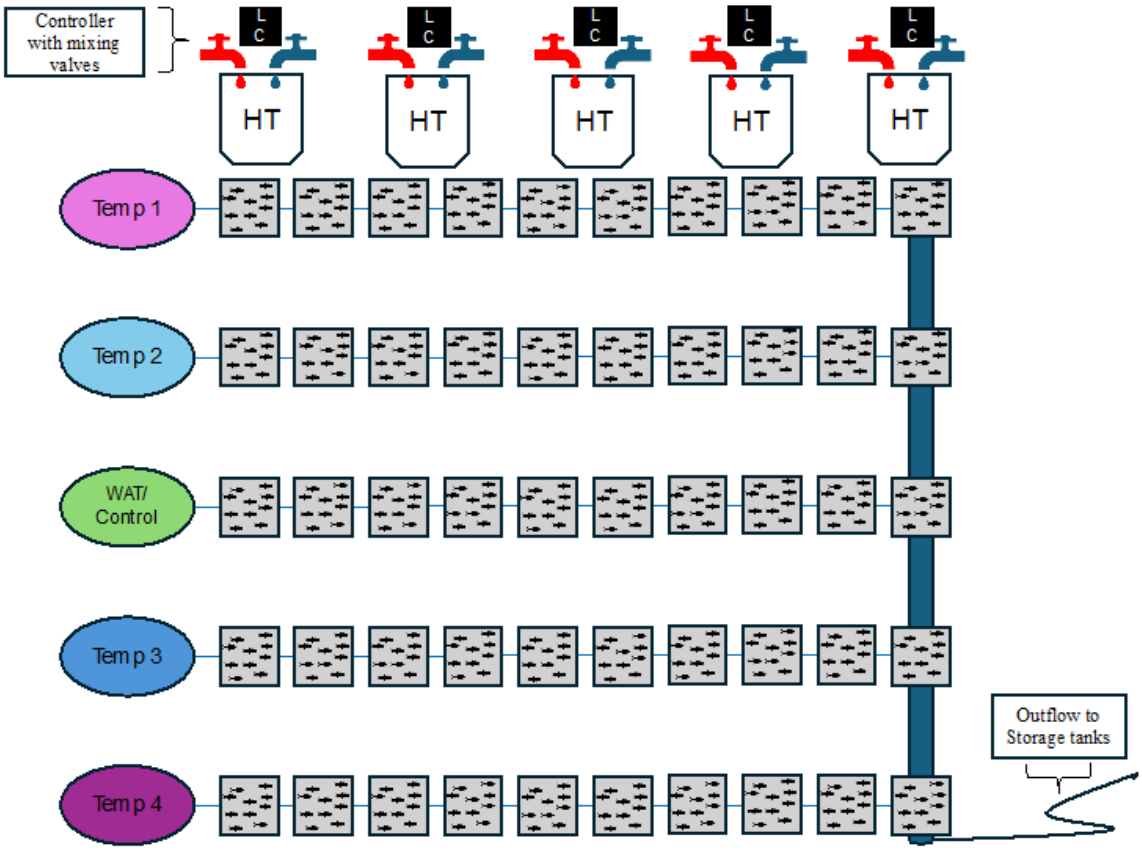


Figure 5.2 Schematic diagram for ILT experimental setup. 5 rows of 10, 2-L tanks were regulated by a temperature controller (LC) connected to two solenoid mixing valves that were plumbed to outside storage tanks. Water entering each tank was gravity fed by one of five head tanks (HT), each corresponding to a different row and temperature treatment. Each row was assigned a static temperature to test larval fish for seven-days of exposure.

Two storage racks were used for CTM trials that held four, 2-L glass tanks (19x19x12cm) each inside the trailer. Water for the tanks was supplied through the ILT tanks system, where ILT rows that had desired temperatures had water diverted into the CTM tanks before trials. To maintain proper DO levels and a homogenous thermal profile for each tank, aeration and stir plates with stir bars were used. The 2-L glass tanks were equipped with B-series Love Controls Division temperature controllers to regulate the rate of temperature change at $\pm$ 0.3°C/min (18°C hr^{-1}) with submersible aquarium heaters (for CTMax) or pumps that passed ice water through a radiator pipe (for CTMin) as recommended by Beitinger et al. (2000; Figure 4.3).

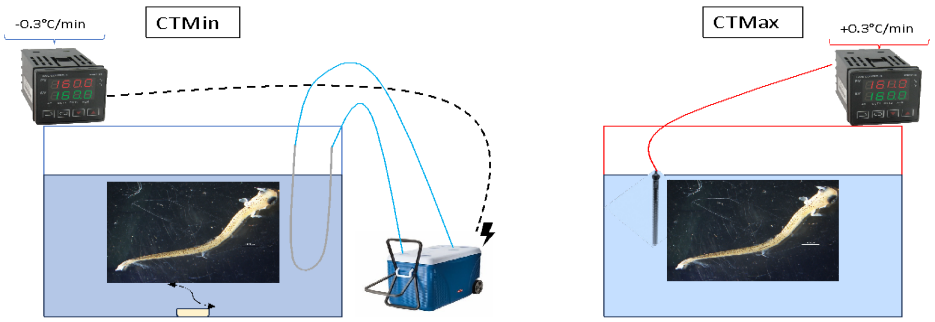


Figure 5.3 Schematic diagram for critical thermal minimum (CTMin) and critical thermal maximum (CTMax) experimental setup. 2-L tanks were regulated by B-series Love Controls Division temperature controllers to control rate of increasing (CTMax) or decreasing temperature (CTMin). CTMin was connected to a submersible pump that allowed cold water to pass through a radiator coil located in the tank decrease the water temperature by 0.3°C min^{-1}. Controllers for CTMax trials were connected to a submersible aquarium heater located in the tank to increase the water temperature by 0.3°C min^{-1}.

5.2.4 *Selection of Weekly Average Temperature*

Temperature treatments were defined by the weekly average temperature (WAT) of the stream one week prior to experiments to reflect the natural stream temperature. The WAT was determined by placing a HOBO logger in proximity of our larval sampling site and calculating the mean of the daily average temperatures for a seven-day period. Since the larvae experienced dynamic temperature diel fluctuations, the weekly mean during the seven-day period reflected their most recent temperature exposures. WAT temperatures were used for CTM trials and temperature above, below, and including the WAT were used for ILT trials as described below.

5.2.5 *Upper Incipient Lethal Trials*

Individual rows of the tank rack were randomly assigned a target temperature (Figure 5.2). One row functioned as the control and maintained the calculated WAT throughout the seven-day exposure. The high and low temperature treatments for each rack were estimated based on the regression model of Riepe et al. (2024). The WAT of the stream for the particular week was used to calculate the highest temperatures the fish may tolerate for the temperature rack. For instance, with a WAT of 20.9°C the highest temperature the fish may tolerate before mortality is predicted to be 32.9°C. The remaining three treatments were distributed between the WAT and the highest temperature. Thus, the treatments for the upper ILT were 20.9ºC (WAT), 23.9°C, 26.9°C, 29.9°C, and 32.9°C.

Upper ILT trials lasted seven days. Larvae were directly transferred from the holding tank using an aquarium net (3.14” x 3.93”) from the holding tank into the UILT tanks once temperatures were set. Each row contained ten tanks and held ten larvae for the duration of the experiment. Once the larvae were transferred into each UILT tank, the larvae were observed for the first 60 minutes to ensure mortality did not occur. Larvae were observed once every eight hours thereafter until the seven-day trials were completed. Temperature was recorded with a calibrated ThermPro thermometer for every tank at each fish check. DO and pH were recorded with YSI ProODO and pH instruments at every fish check, from end tanks in opposite rows. If a mortality occurred, larvae were removed from the tank, measured for total length (mm), and then preserved at 95% EtOH for later fish identification and determination of development stage. At

the end of a seven-day trial, any surviving larvae were euthanized with tricaine methanolsulfate (MS-222; Western Chemicals), measured, and preserved for further identification.

5.2.6 *Critical Thermal Maximum and Minimum*

The CTMax and CTMin trials followed the methods as described by Beitinger et al. (2000) for individual fish. Prior to CTM trials, all larvae were held for at least one day in a holding tank that received water from the stream. The starting temperature for both CTM experiments was the calculated WAT for the given sampling period when the larvae were first collected from the stream.

After the 24 hour holding period, one larval fish was transferred into one, 2-L glass tank to undergo the temperature test. Water temperature increased or decreased at a rate of 0.3°C min^{-1} until loss of equilibrium (LOE) was observed; defined as the failure to uphold dorsal-ventral orientation (Bennett and Beitinger 1997; Selong et al. 2001; Carveth et al. 2006). Once LOE was observed larvae were transferred into a recovery tank at the original starting WAT temperature for 20 minutes and observed for any post-trial mortality (Beitinger et al. 2000; Cadmus et al. 2014). CTM trials are not intended to cause mortality and therefore any larvae that did not survive during the trial or recovery period were not included in the data analysis (Beitinger et al. 2000). Start and end temperatures were recorded with a ThermPro thermometer and DO and pH were recorded with YSI ProODO and pH instruments. All larval fish at the end of experiments were euthanized with MS-222, total length was recorded (mm), and fish were preserved in 95% EtOH for larval identification and determination of development stage at a later date.

5.2.7 *Larval Fish and Stage Identification*

Post-experiment larvae were identified by species and development stage was determined using an Olympus SZX7 Fluorescence Stereo Microscope. Larvae were transferred from their individual preserved container onto a glass petri dish with 100% EtOH. A translucent millimeter ruler was placed between the petri dish and microscope stage to record post-experiment total length and standard length.

We identified the fish to species following the protocol recommended by Snyder et al. (2004; 2016). Morphometric features such as snout-to-vent length and position of mouth were used to distinguish larvae to family level between the Flannelmouth and Bluehead Sucker and Roundtail Chub. The two catostomids in the study were identified by species using a list of both meristic and qualitative attributes consisting of dorsal-fin-ray counts, dorsal and ventral pigmentation patterns, total length, and size when developmental features appear (e.g., presence of dorsal-fin and caudal-fin rays, development of pelvic fin buds, and gut development stage). White Suckers (*Catostomus commersonii*) and consequent hybrids or non-study cyprinids such as Speckled Dace (*Rhinichthys osculus*) or Redfin Shiners (*Lythrurus umbratilis)* were also present in Roubideau Creek during our fish collection, thus similar identification criteria were used to exclude the non-test species from the post-experiment sample. White Suckers were differentiated from both Bluehead Suckers and Flannelmouth Suckers by a distinct continuous line of pigmentation found ventrally on the larvae along with an organized dorsal pigmentation pattern. Hybrids of the three catostomids were identified by displaying a mixture of pigmentation patterns, ray counts, and size at development that are characteristic of White Sucker and crossed native catostomids. Chub were separated from other small-bodied cyprinids by size at development and myomere counts. In general, Roundtail Chub larvae are consistently larger at

all larval stages and less developed than non-test cyprinid species, along with having a higher density of myomeres total (Snyder et al. 2016).

5.2.8 *Water Chemistry*

Water samples were collected to understand other environmental variables that may influence temperature tolerance. Water samples were analyzed for the presence of heavy metals and trace metals (e.g., aluminum, arsenic, cadmium, copper, iron, magnesium, lead, selenium, zinc, calcium, potassium, manganese, and sodium) and nutrients (e.g., phosphorus, nitrate, nitrite, chloride, ammonia, and sulfate). We followed protocols from Colorado River Watch Laboratory and Colorado Parks and Wildlife Toxicology Laboratory. Water was collected from Roubideau Creek, Potter Creek, and the storage tanks with 200 mL Nalgene® bottles. A non-filtered sample was collected in a 60 mL sample bottle for total metal analysis and a filtered sample was collected for dissolved metals using a 45 µm filter on a 60 mL syringe. Both samples for metals were preserved with six drops of 3% high-purity, nitric acid. Two non-filtered water samples for nutrients were collected in 15 mL Falcon® tubes and placed on ice to remain cold. One of the two nutrient samples was preserved with 3% sulfuric acid. Samples were analyzed within two months of collection.

Metal concentrations were analyzed using a ThermoScientific iCAP 6000 Series Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). The samples were introduced via a CETAC ultrasonic nebulizer (U5000AT+) and operated with iTeva software (version 2.8.0.97). The nebulizer was set to temperatures of 3°C and 140°C. Samples were injected into the plasma using a quartz torch with a 2.0 mm quartz center tube. Before analysis, nitrogen gas (N2) was used to purge the instrument for at least five hours, followed by the ignition of high-purity argon (Ar) plasma. The plasma was stabilized for one hour prior to initial calibration. Calibration was achieved using a five-point curve to encompass the analyte concentrations (Table 5.1). Matrix solutions for blanks and standard dilutions were prepared in 1% HNO3 and 1% HCl using deionized water (DI; Barnstead Nanopure system). Calibration standards were sourced from NIST-Traceable certified standards (SRM 3100 Series). Yttrium (Y) was used as an internal standard, introduced inline and monitored at three wavelengths (Table 5.1). Method detection and reporting limits were established following EPA procedures.

Table 5.1 Calibration standards for ICP-OES method to detect aluminum (Al), arsenic (As), calcium (Ca), cadmium (Cd), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), lead (Pb), selenium (Se), and zinc (Zn). All units are represented in μg L^{-1}. Method detection limits (MDL), reporting limits (RL), and axial or radial detection are included for each element.

Analyte	Wavelength	Blank	STD1	STD2	STD3	STD4	STD5	STD6	MDL	RL	Y	Axial/Radial
Al	396.15	0	50	500	1,000	2,500	5,000	50,000	9.90	40.6	371.03	Radial
As	189.04	0	15	150	300	750	1,500	-	0.85	3.50	224.31	Axial
Ca	315.89	0	2,000	20,000	40,000	100,000	200,000	500,000	38.1	156	360.07	Radial
Cd	214.44	0	5	50	100	250	500	-	0.25	1.02	244.31	Axial
Cu	327.40	0	5	50	100	250	500	-	1.35	5.54	360.07	Axial
Fe	238.20	0	50	500	1,000	2,500	5,000	-	6.13	25.1	360.07	Axial
Fe	274.93	0	50	500	1,000	2,500	5,000	20,000	6.13	25.1	360.07	Axial
K	766.49	0	500	5,000	10,000	25,000	50,000	-	15.1	62.1	371.03	Radial
Mg	279.08	0	-	10,000	-	50,000	100,000	300,000	14.5	59.4	360.07	Radial
Mg	285.21	0	1,000	10,000	20,000	50,000	100,000	-	14.5	59.4	360.07	Radial
Mn	257.61	0	50	500	1,000	2,500	5,000	-	0.60	2.46	360.07	Axial
Na	589.59	0	500	5,000	10,000	25,000	50,000	400,000	18.3	74.9	371.03	Radial
Pb	220.35	0	5	50	100	250	500	-	1.30	5.31	224.31	Axial
Se	196.09	0	-	10	20	50	100	-	1.88	7.71	244.31	Axial
Zn	213.86	0	50	500	1,000	2,500	5,000	-	1.41	5.79	244.31	Axial

5.2.9 *Statistical Analyses*

5.2.9.1 *Upper Incipient Lethal Tests*

A Kaplan-Meier survival model (Therneau 2024) was used to estimate the probability of survival for larval three species in UILTs. Survival was modeled over the seven-day trial for a total of 176 hours. A log-rank test was used to determine if a difference between the probabilities of survival between temperature treatments was detected. Significance was set at 0.05 (α). Analyses were conducted in program R (version 4.3.1) using the *survival* and *survminer* packages.

An analysis of variance (ANOVA) with unbalanced design was used to detect any differences in percent larval survival at the end of the experiment among the UILT temperature treatments. One-way ANOVA was used to separately test if DO and pH levels varied when treating the UILT rows as random variables. If differences were detected in the ANOVA tests, Tukey's Honest Significance test was then used for multiple comparisons. All ANOVA tests were set to a significance of 0.05 (α).

5.2.9.2 *Critical Thermal Maximum*

To determine whether the LOE among Bluehead Suckers was a factor of WAT in CTMaximum trials, an ANOVA test with unbalanced design was used. The ANOVA test was set to a significance of 0.05 (α). Few trials for all other CTMax and CTMin tests were conducted, thus statistical tests were not used.

5.3 Preliminary Results

5.3.1 *Stream Conditions and WAT*

During the 2024 spawning and larval rearing season (April-June), temperatures varied between 4.5–28.1ºC in Roubideau Creek (Figure 5.4). WAT for the first week of trials was 16.7ºC while the WAT for the second week of trials was 20.9ºC. Maximum temperatures were 20.3ºC and 25.6ºC for the first and second week respectively and minimum temperatures were 13.6ºC and 15.4ºC (Figure 5.5). Daily temperature fluctuations were approximately 6.9ºC and weekly fluctuations were 11.1ºC.

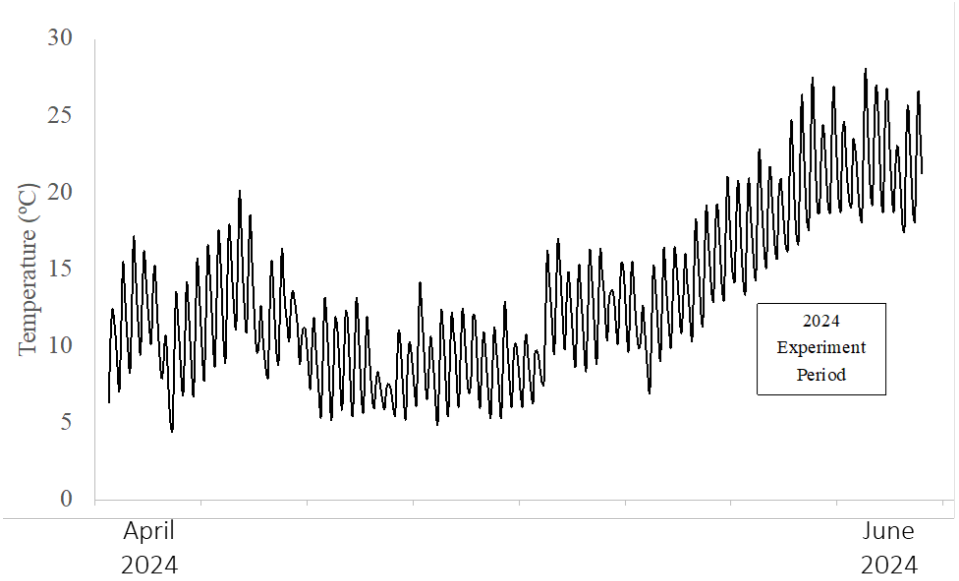
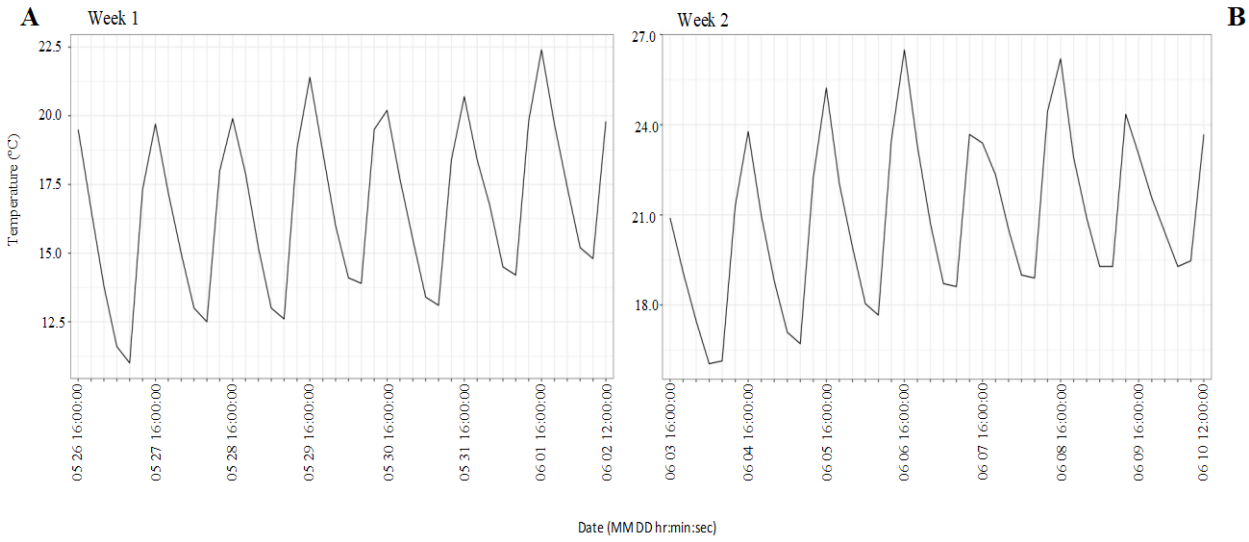


Figure 5.4 2024 temperature (°C) data for Roubideau Creek from April 2024 to the end of the field experiments in June 2024.



C

Summary Temperature (ºC)	Week 1: (5/26/24 to 6/2/24)	Week 2: (6/3/24 to 6/10/24)
Minimum Observed	13.6ºC	15.4ºC
WAT	16.7ºC	20.9ºC
Maximum Observed	20.3ºC	26.5ºC

Figure 5.5 A) Roubideau Creek temperature profiles for week one (5/26/2024 – 6/2/2024) and B) week two (6/3/2024 – 6/10/2024) of trials. HOBO logger readings were set to record every four hours. C) Summary temperatures for weeks one and two during the 2024 field season. Minimum and maximum temperatures (°C) are provided in addition to weekly average temperature (WAT).

5.3.2 *Water Chemistry*

Water samples were collected from both Roubideau Creek and Potter Creek on four different occasions in 2024. Water hardness ranged from 116-207 mg/L in Roubideau Creek and 136-331 mg/L in Potter Creek. In Roubideau Creek, aluminum exceeded chronic standards set by CDPHE during one sampling occasion (840.5 µg total recoverable Al/L) when the water hardness was 125 mg/L (Table 5.2).

Table 5.2 Results of Roubideau Creek dissolved and total metal concentrations using ICP-OES method for May 20th, May 28th, June 3rd, and June 17th, 2024. Concentrations are listed as mg L^{-1}. No concentrations are listed for detections less than the method detection limit (<MDL). Asterisks (** **) around individual numbers signify exceedance of chronic standards set by the Colorado Department of Public Health and Environment.

Date	**20-May-24**		**28-May-24**		**3-Jun-24**		**17-Jun-24**	
Water Location	**Roubideau Creek**		**Roubideau Creek**		**Roubideau Creek**		**Roubideau Creek**	
Element	**Total**	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**
Al	**840.5**	21.13	548.10	10.51	540.50	<MDL	68.48	<MDL
As	<MDL	1.20	1.18	<MDL	1.99	<MDL	1.67	<MDL
Ca	33870	32680	32990.00	32910	43050	37860	60050.00	60700.00
Cd	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Cu	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Fe	428.20	8.72	312.10	8.97	399.70	<MDL	50.68	<MDL
K	2156	1945	1753.00	1684	2215	2073	3177.00	3191.00
Mg	9911	9480	8190.00	8330	10240	9325	12990.00	13470.00
Mn	24.60	7.06	15.57	4.78	58.56	9.27	14.24	9.07
Na	12120	11910	15170.00	15650	24310	24440	63570.00	63000.00
Pb	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Se	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Zn	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Hardness (mg/L)	125	121	116	116	150	133	203	207

No exceedances were detected thereafter by aluminum or any other metals in Roubideau Creek. Metals in Potter Creek did not exceed acute or chronic standards (Table 5.3).

Table 5.3 Results of Potter Creek dissolved and total metal concentrations using ICP-OES method for May 20th, May 28th, June 3rd, and June 17th, 2024. Concentrations are listed as mg L^{-1}. No concentrations are listed for detections less than the method detection limit (<MDL).

Date	**20-May-24**		**28-May-24**		**3-Jun-24**		**17-Jun-24**	
Water Location	**Potter Creek**		**Potter Creek**		**Potter Creek**		**Potter Creek**	
Element	**Total**	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**	**Total**	**Dissolved**
Al	461.40	19.25	35.23	10.57	218.60	<MDL	11.65	<MDL
As	<MDL	1.02	<MDL	<MDL	1.18	0.96	<MDL	0.96
Ca	36260	36540	53940	54750	69890	68820	79200.00	79920.00
Cd	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Cu	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Fe	212.10	15.93	32.85	11.29	229.20	<MDL	37.39	<MDL
K	2246	2205	2917	2933	4634	4618	5054.00	5186.00
Mg	11030	11140	17160	17370	26390	26090	31250.00	31860.00
Mn	11.01	6.71	8.61	8.22	36.14	14.49	4.75	3.32
Na	13090	13180	23490	23630	46100	46460	62720.00	62490.00
Pb	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Se	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Zn	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Hardness (mg/L)	136	137	205	208	283	279	326	331

Two water samples were collected from both the cold and hot storage tanks to estimate metal presence within the UILT racks. Water hardness in the storage tanks ranged from 192-198 mg/L. No metals exceeded CDPHE standards. Copper in the cold storage tanks was near the exceedance threshold (Table 5.4) and was likely caused by the Frigid Units immersion chiller, which uses an exposed copper coil when submerged.

Table 5.4 Results of the cold and hot storage tanks dissolved and total metal concentrations using ICP-OES method for June 10th and June 17th, 2024. Concentrations are listed as mg L^{-1}. No concentrations are listed for detections less than the method detection limit (<MDL).

Date	10-Jun-24	10-Jun-24	17-Jun-24	17-Jun-24
Water Location	Cold Storage	Cold Storage	Hot Storage	Hot Storage
Element	Total	Dissolved	Total	Dissolved
Al	11.38	44.36	16.34	72.63
As	0.43	0.89	1.59	<MDL
Ca	59780.00	59520.00	52310.00	53520.00
Cd	<MDL	<MDL	<MDL	<MDL
Cu	6.49	7.64	23.31	29.40
Fe	-4.87	<MDL	-4.46	<MDL
K	3241.00	3181.00	3807.00	3823.00
Mg	11810.00	11750.00	14970.00	15240.00
Mn	0.43	2.87	2.64	<MDL
Na	62480.00	62040.00	39400.00	39610.00
Pb	0.08	<MDL	<MDL	<MDL
Se	<MDL	<MDL	<MDL	<MDL
Zn	26.07	31.66	44.52	67.88
Hardness (mg/L)	198	197	192	196

5.3.3 *Upper Incipient Lethal Tests*

A total of 498 larvae were collected and used for UILTs. Unfortunately, we were unable to achieve initial row temperatures thus post-hoc tank temperature treatments were assigned by average tank temperature as the following: 19ºC (n = 10 tanks), 24ºC (n = 3 tanks), 25ºC (n = 10 tanks), 26ºC (n = 9 tanks), 27ºC (n = 10 tanks), 28ºC (n = 8 tanks). The above temperature treatments will be referred to throughout the rest of the study. Survival within each tank is reported as total tank survival and not by individual species. Species identification is currently in progress.

DO levels among tanks ranged from 5.05 to 10.39 mg L^{-1} and were significantly different between rows ($p < 0.05$, $F_{4,105} = 45.39$; Figure 5.6).

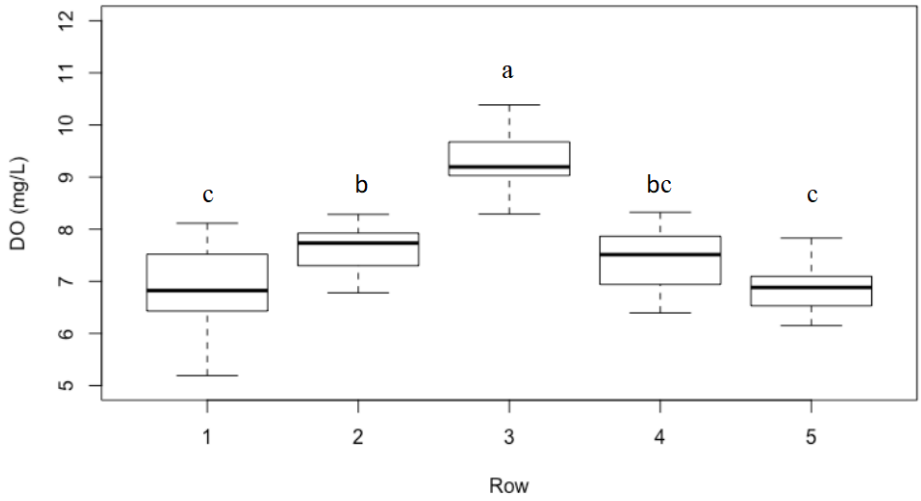


Figure 5.6 Dissolved oxygen levels (mg L^{-1}) for UILT rows in 2024 trial. Boxplot outer edges represent the upper quartile (75th percentile; top) and lower quartile (25th percentile; bottom); the median (50th percentile) is represented by the darkened horizontal band; whiskers edge indicate lowest to highest data. Letters above the boxplot represent results of the multiple comparison test. Matching letters indicate non-significant relationship between two or more rows.

No significant differences were observed for pH among rows ($p = 0.969$, $F_{4,105} = 0.136$; Figure 5.7).

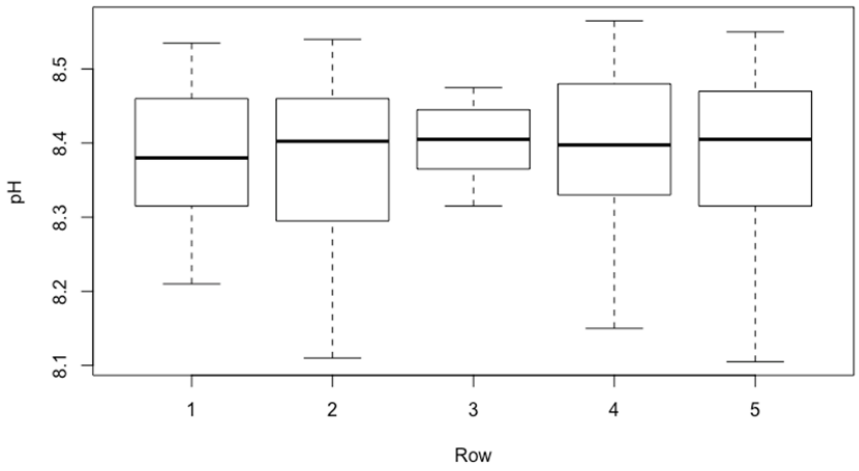


Figure 5.7 pH levels for UILT trials by row in 2024 trial. Boxplot outer edges represent the upper quartile (75th percentile; top) and lower quartile (25th percentile; bottom); the median (50th percentile) is represented by the darkened horizontal band; whiskers edge indicate lowest to highest data. No significant differences were detected between rows.

A Kaplan-Meier survival curve was used to estimate larval survival curves across the six temperature treatments over time (Figure 5.8). Significant differences were detected between survival curves ($p < 0.05$, $X^2_{5,1186} = 24$). However, mortalities were low and we never detected less than 50% survival in any temperature treatments. At the end of the experiment (176 hours), the estimated probability of survival was 0.93 ± 0.02 for 19ºC, 0.93 ± 0.04 for 24ºC, 0.88 ± 0.06 for 25ºC, 0.84 ± 0.04 for 26ºC, 0.56 ± 0.18 for 27ºC, and 0.70 ± 0.14 for 28ºC.

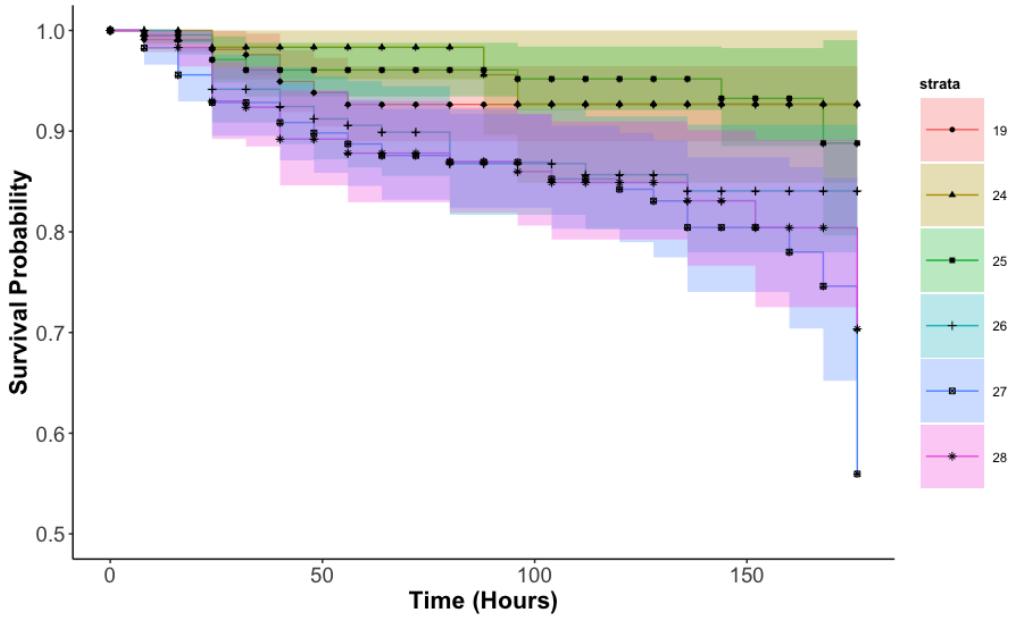


Figure 5.8 Kaplan-Meier survival curve for larvae collected in Roubideau Creek that underwent the UILT trials in 2024 as a function of total hours over the experiment. Different temperature treatments (strata) are represented by point type and color shaded around the individual lines (19ºC-red ●, 24ºC-yellow ▲, 25ºC-green ■, 26ºC-turquoise +, 27ºC-blue ☒, 28ºC-pink ✳). Shaded areas illustrate 95% confidence intervals of modeled data.

Temperature treatment influenced the end proportion of survival in the UILT trial ($p < 0.05$, $F_{4,44} = 4.146$; Figure 4.9). Proportion of survival was highest in the 24ºC treatment and lowest in the 27ºC treatment. Comparisons between treatments indicated that 19:27ºC ($p = 0.03$) and 25:27ºC ($p = 0.01$) were statistically different. The larvae in the 27ºC treatments indicated a lower proportion of survival than the 19ºC and 25ºC treatments. All other comparisons were found to have similar proportions of survival ($p > 0.05$).

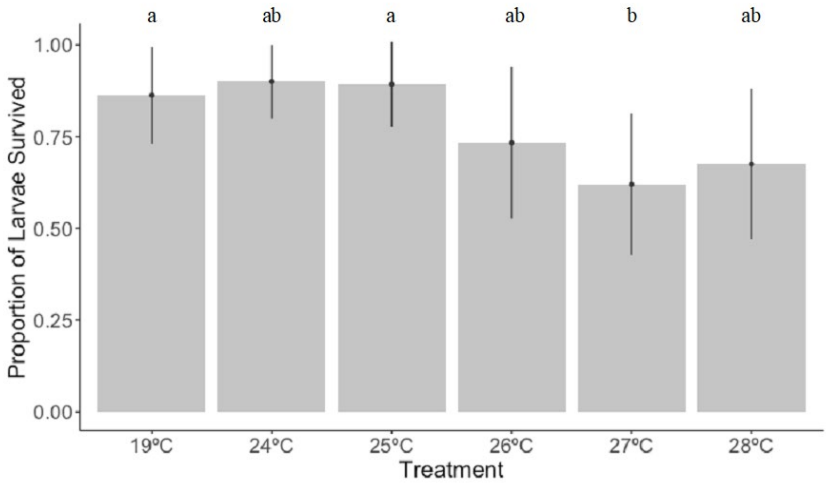


Figure 5.9 Barplots representing proportion of larval survival by temperature treatment of the UILT trials in 2024. Vertical lines symbolize the standard deviation. Letters above the barplot represent results of the multiple comparison test. Matching letters indicate non-significant relationship between two or more treatments.

5.3.4 *Critical Thermal Maximum and Critical Thermal Minimum*

We conducted 61 trials for CTMax and 53 trials for CTMin using three species larvae. DO and pH levels after each trial varied by an average of 1.92 $\pm$ 0.33 mg L^{-1} for CTMax, and 2.58 $\pm$ 0.15 mg L^{-1} for CTMin. Throughout the trial period, nine larvae did not recover after the CTMax trials, and five larvae did not recover after the CTMin trials. These larvae were excluded from the analyses and results.

We tested nine Bluehead Suckers (BHS) and 11 Flannelmouth Suckers (FMS) at 16.7ºC WAT and 30 Roundtail Chub (RTC) at 20.9ºC for CTMin trials. Mean LOE temperature for BHS larvae was 7.9 + 0.9 ºC, while FMS larvae held an LOE mean temperature of 8.9 $\pm$ 1.4 ºC (Figure 5.10). RTC larvae collected showed an LOE mean temperature of 7.0 $\pm$ 0.2 ºC (Figure 5.10F).

We tested eight FMS at 16.7ºC WAT, 31 RTC at 20.9ºC WAT, and 21 BHS at both 16.7ºC (n = 10) and 20.9ºC (n = 11) for CTMax trials. Mean LOE temperature for FMS at 16.7ºC WAT was 32.2 $\pm$ 1.8 ºC. RTC at 20.9ºC WAT had a mean LOE temperature of 33.6 ± 0.4 ºC (Figure 4.10C; Figure 4.10E). BHS larvae's mean LOE was 33.6 ± 0.7 ºC and 33.9 ± 0.88 ºC for WAT of 16.7ºC and 20.9ºC, respectively (Figure 4.10A). BHS larvae collected at the two WATs did not show a significant difference in mean LOE temperature ($p = 0.65$, $F_{1,19} = 0.21$).

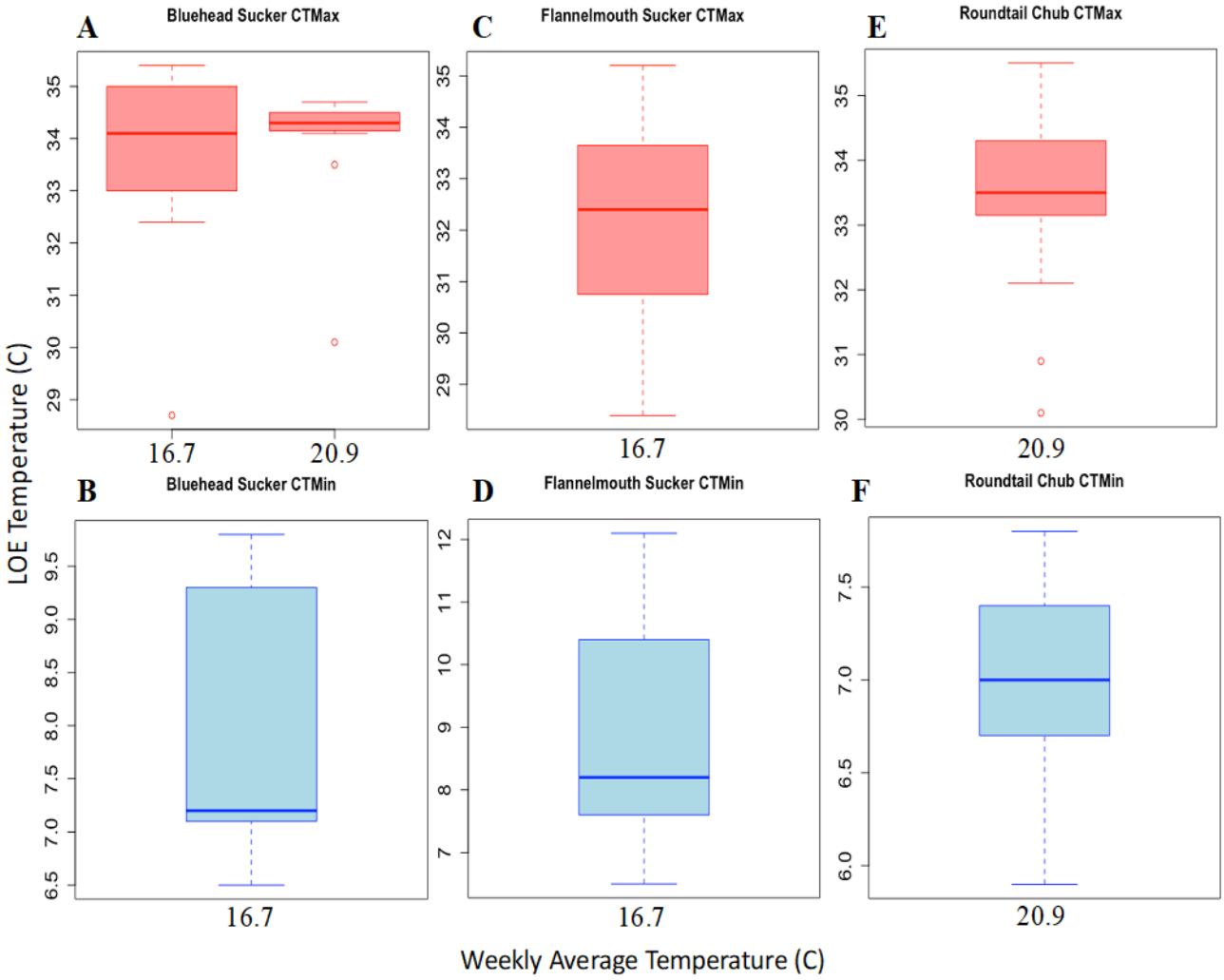


Figure 5.10 Loss of equilibrium (LOE) of Bluehead Sucker (A,B), Flannelmouth Sucker (C,D), and Roundtail Chub (E,F) for critical thermal maximum (red) and critical thermal minimum (blue) at weekly average temperatures (°C). Boxplot outer edges represent the upper quartile (75th percentile; top) and lower quartile (25th percentile; bottom); the median (50th percentile) is represented by the darkened horizontal band; whiskers edge indicate lowest to highest LOE data.

5.4 Discussion and Future Work

Our preliminary results provide information for field-derived temperature tolerances for three species larvae. To our understanding, this study is currently the only one that has attempted to collect larval Bluehead Sucker, Flannelmouth Sucker, and Roundtail Chub CTM and ILT data by using laboratory tests in the field. We determined that for Bluehead Sucker exposed to average weekly temperatures of 16.7°C, LOE ranged from 7.9°C $\pm$ 0.9°C to 33.6 $\pm$ 0.2 ºC. Likewise, Bluehead Suckers exposed to an average weekly temperature of 20.9°C, the maximum temperature they could tolerate was similar at 33.9 $\pm$ 0.9°C. Similar studies in the laboratory with a controlled acclimation temperature of 18°C suggest a maximum temperature tolerance of 32.1 $\pm$ 2.4°C (Riepe et al. 2023) which is lower than the two temperatures tested thus far in this study. Additionally, Flannelmouth Suckers collected when the weekly average temperature was 16.7°C showed a temperature tolerance range from CTM trials of 8.9 $\pm$ 1.4°C to 32.2 $\pm$ 1.8 °C. Compared to laboratory-derived temperature ranges at an acclimation temperature of 16°C,

Riepe et al. (2024) indicated a tolerance range of 7.2 $\pm$ 0.5°C to 32.6 $\pm$ 0.4°C. Interestingly the lower tolerance range at 16.7°C was lower in laboratory studies than field-derived studies. Both cases of Bluehead Sucker and Flannelmouth Sucker may indicate the potential for over protected or under protected temperature standards when developed in the laboratory, but more data needs to be collected to better understand this relationship and determine if temperature standards should be developed in the laboratory or with laboratory-based methods in the field.

The UILT data suggest that the temperatures we used may not be lethal temperatures for the fish. The, LT_{50} (50% mortality within a treatment) was not reached in any of our trials. LT_{50} typically serves as an important metric in laboratory studies that classifies the end of a trial where half of the population would not survive (Fry 1947; Bennett and Beitinger 1997). However, currently our data includes all the three species together since we are still working to identify the fish thus, survival estimates may change when we account for individual species survival rather than tank survival.

Another field season will start in the spring of 2025 and will include more ILT and CTM trials at different WAT temperatures to complete our dataset.

5.5 References

Bates, A. E., and S. A. Morely. 2020. Interpreting empirical estimates of experimentally derived physiological and biological thermal limits in ectotherms. Canadian Journal of Zoology 98(4): 237-244.

Becker, C. D. and R. G. Genoway. 1979. Evaluation of the critical thermal maximum for determining temperature tolerance of freshwater fish. Environmental Biology of Fishes 4: 245-256.

Beitinger, T. L., W. A. Bennett, and R. W. McCauley. 2000. Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. Environmental Biology of Fishes 58: 237-275.

Bennett, W. A. and T. L. Beitinger. 1997. Temperature tolerance of the Sheepshead Minnow, *Cyprinodon Variegatus.* Copeia 1997: 77-87.

Bezzerides, N., and K. Bestgen. 2002. Status review of Roundtail Chub *Gila robusta*, Flannelmouth Sucker *Catostomus latipinnis*, and Bluehead Sucker *Catostomus discobolus* in the Colorado River Basin. Technical Report: Larval Fish Laboratory Contribution 118.

Brungs, W. A. and B. R. Jones. 1977. Temperature criteria for freshwater fish: protocol and procedures. EPA-600/3-77-061. Environmental Research Laboratory, Duluth, Minnesota.

Cadmus, P., A. L. Jefferson, and A. Townsend. 2014. Water pollution studies, annual progress report. Colorado Parks and Wildlife Annual Report.

Carveth, C. J., A. M. Widmer, and S. A. Bonar. 2006. Comparison of upper thermal tolerances of native and nonnative fish species in Arizona. Transactions of the American Fisheries Society 135: 1433-1440.

Cech, J. J. and P. B. Moyle. 2004. Fishes an introduction to ichthyology. Prentice Hall, New Jersey.

Chen, Z, A. P. Farrell, A. Matala, N. Hoffman, S. R. Narum. 2018. Physiological and genomic signatures of evolutionary thermal adaptation in redband trout from extreme climates. Evolutionary Applications 11(9): 1686-1699.

Compton, R. I., W. A. Hubert, F. J. Rahel, M. C. Quist, and M. R. Bower. 2008. Influences of fragmentation on three species of native warmwater fishes in a Colorado River Basin headwater stream system, Wyoming. North American Journal of Fisheries Management 28: 1733-1743.

Coutant, C. C. 1977. Compilation of temperature preference data. Journal of Fisheries Research Board of Canada 34: 739-745.

Currie, R. J., W. A. Bennett, and T. L. Beitinger. 1998. Critical thermal minima and maxima of three freshwater game-fish species acclimated to constant temperatures. Environmental Biology of Fishes 51: 187-200.

Dahlke, F. T., S. Wohlrab, M. Butzin, and H. O. Pörtner. 2020. Thermal bottlenecks in the life cycle define climate vulnerability of fish. Science 369: 65-70.

Desforges, J. E., K. Birnie-Gauvin, F. Jutfelt, K. M. Gilmour, E. J. Eliason, T. L. Dressler, D. J. McKenzie, A. E. Bates, M. J. Lawerence, N. Fangue, S. J. Cooke. 2023. Journal of Fish Biology 102(5): 1000-1016.

De Vries, P., J. E. Tamis, A. J. Murk, and M. G. D. Smit. 2008. Development and application of a species sensitivity distribution for temperature-induced mortality in the aquatic environment. Environmental Toxicology and Chemistry 27(12): 2591-2598.

Dibble, K. L., C. B. Yackulic, T. A. Kennedy, K. R. Bestgen, and J. C. Schmidt. 2021. Water storage decisions will determine the distribution and persistence of imperiled river fishes. Ecological Applications 31(2): e02279.

Donaldson, M. R., S. J. Cooke, D. A. Patterson, and J. S. Macdonald. 2008. Cold shock and fish. Journal of Fish Biology 73: 1491-1530.

Dudley, R. K. and S. P. Platania. 2007. Flow regulation and fragmentation imperil pelagic-spawning riverine fishes. Ecological Applications 17: 2074-2086.

EPA. 1986. Quality criteria for water. EPA 440/5-86-001.

EPRI. 2011. Thermal toxicity literature evaluation. EPRI, Palo Alto, CA: 1023095

Fry, F. E. J. 1947. Effects of the environment on animal activity. The University of Toronto Press, Toronto.

Handeland, S. O., A. Imsland, and S. O. Stefansson. 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic Salmon *Salmo salar* post-smolts. Aquaculture 283(1): 36-42.

Hasnain, S. S., M. D. Escobar, and B. J. Shuter. 2018. Estimating thermal response metrics for North American freshwater fish using Bayesian phylogenetic regression. Canadian Journal of Fisheries and Aquatic Sciences 75: 1878-1885.

Jones, N. E. and I. C. Petreman. 2013. Relating extremes of flow and air temperature to stream fish communities. Ecohydrology 6(5): 826-835.

Jonsson, B. 2023. Thermal effects on ecological traits of salmonids. Fishes 8(337): 1-20.

Keller, A. A., and G. Klein-MacPhee. 2000. Impact of elevated temperature on the growth, survival, and trophic dynamics of winter Flounder larvae: A mesocosm study. Canadian Journal of Fisheries and Aquatic Sciences 57(12): 2382-2392.

Killen, S. S. 2014. Growth trajectory influences temperature preference in fish through an effect on metabolic rate. Journal of Animal Ecology 83: 1513-1522.

Konecki, J. T., C. A. Woody, and T. P. Quinn. 1995. Critical thermal maxima of Coho Salmon (*Oncorhynchus kisutch)* fry under field and laboratory acclimation regimes. Canadian Journal of Zoology 73: 993-996.

Li, D., M. Dorber, V. Barbarossa, and F. Verones. 2022. Global characterization factors for quantifying the impacts of increasing water temperature on freshwater fish. Ecological Indicators 142: 109201.

Lutterschmidt, W. I., and V. H. Hutchison. 1997. The critical thermal maxima: History and critique. Canadian Journal of Zoology 75: 1561-1574.

Mandeville, C. P., F. J. Rahel, L. S. Patterson, and A. W. Walters. 2019. Integrating fish assemblage data, modeled stream temperatures, and thermal tolerance metrics to develop thermal guilds for water temperature regulation: Wyoming case study. Transactions of the American Fisheries Society 148: 739-754.

Payne, N. L., J. A. Smith, D. E. van der Meulen, M. D. Taylor, Y. Y. Watanbe, A. Takahashi, T .A. Marzullo, C. A. Gray, G. Cadiou, I. M. Suthers. 2016. Temperature dependence of fish performance in the wild: Links with species biogeography and physiological thermal tolerance. Functional Ecology 30(6): 903-912.

Regonda, S. K., B. Rajagopalan, M. Clark, and E. Zagona. 2006. A multimodel ensemble forecast framework: Application to spring seasonal flows in the Gunnison River Basin. Water Resources Research 42: W09404.

Reynolds, L. V., P. B. Shafroth, and N. L. Poff. 2015. Modeled intermittency risk for small streams in the Upper Colorado River Basin under climate change. Journal of Hydrology 523: 768-780.

Riepe, T. B., Z. Hooley-Underwood, R. E. McDevitt, A. Sralik, and P. Cadmus. 2023. Increased density of Bluehead Sucker *Catostomus discobolus* larvae decreases critical thermal maximum. North American Journal of Fisheries Management 43: 1135-1142.

Riepe, T. B., Z. E. Hooley-Underwood, and M. Johnson. 2024. Thermal tolerance of larval Flannelmouth Sucker *Catostomus latipinnis* acclimated to three temperatures. Fishes 9: 181.

Rodnick, K. J., A. K. Gamperl, K. R. Lizars, M. T. Bennett, R. N. Rausch, and E. R. Keeley. 2004. Thermal tolerance and metabolic physiology among redband trout populations in south-eastern Oregon. Journal of Fish Biology 64: 310-335.

Schofield, P. J., W. F. Loftus, R. M. Kobza, M. I. Cook, and D. H. Slone. 2009. Tolerance of nonindigenous cichlid fishes (*Cichlasoma urophthalmus, Hemichromis letourneuxi)* to low temperature: laboratory and field experiments in south Florida. Biological Invasions 12: 2441-2457.

Selong, J. H., T. E. McMahon, A. V. Zale, and F. T. Barrows. 2001. Effect of temperature on growth and survival of Bull Trout, with application of an improved method for determining thermal tolerance in fishes. Transactions of the American Fisheries Society 130: 1026-1037.

Snyder, D. E., R. T. Muth, and C. L. Bjork. 2004. Catostomid fish larvae and early juveniles of the Upper Colorado River Basin – morphological descriptions, comparisons, and computer-interactive key. Colorado Division of Wildlife, Technical Publication DOW-R-T-42-04.

Snyder, D. E., S. C. Seal, J. A. Charles, and C. L. Bjork. 2016. Cyprinid fish larvae and early juveniles of the Upper Colorado River Basin – morphological descriptions, comparisons, and computer-interactive key. Colorado Parks and Wildlife, Technical Publication DOW-R-T-47-16.

Souchon, Y., and L. Tissot. 2012. Synthesis of thermal tolerances of the common freshwater fish species in large Western European Rivers. Knowledge and Management of Aquatic Ecosystems 405-3.

Therneau, T. M. 2024. A package for survival analysis in R. R package version 3.7-0 https://CRAN.R-project.org/package=survival

Todd, A. S., M. A. Coleman, A. M. Konowal, M. K. May, S. Johnson, N. K. M. Vieira, and J. F. Saunders. 2008. Development of new water temperature criteria to protect Colorado's fisheries. Fisheries 33(9): 433-443.

Udall, B. and J. Overpeck. 2017. The twenty-first century Colorado River hot drought and implications for the future. Water Resources Research 53: 2404-2418.

Volkoff, H. and I. Rønnestad. 2020. Effects of temperature and digestive processes in fish. Temperature 7(4): 207-320.

Wehrly, K. E., L. Wang, and M. Mitro. 2007. Field-based estimates of thermal tolerance for trout: Incorporating exposure time and temperature fluctuation. Transactions of the American Fisheries Society 136: 365-374.

WQCC (Water Quality Control Commission). 2011. Temperature criteria methodology. Policy Statement06-1. Colorado Department of Public Health and Environment.

Pathogenesis of *Renibacterium salmoninarum* in Chinook Salmon following intraperitoneal injection: Description of disease progression following qPCR and histopathology

Riley Dils (CSU Research Associate), Dr. Tawni B. R. Firestone

This project is a collaborative effort with Dr. Eric Fetherman (CPW), Dr. Dana Winkelman (CSU), and Dr. Paula Schaffer (CSU).

6.1 Introduction

Bacterial kidney disease (BKD) is a severe disease among salmonids caused by the Gram-positive bacterium *Renibacterium salmoninarum*. Despite extensive research and use of treatment options such as antimicrobials and vaccination (Evelyn et al. 1986; Griffiths et al. 1998), reported BKD cases have steadily risen in the United States (Riepe et al. *In Review*). From 1996 to 2015, the US Fish and Wildlife Service reported over 8,692 *R. salmoninarum* detections using enzyme-linked immunosorbent assays (ELISA) indicating a previous or current infection and 2,215 cases using qPCR indicating a current infection. High numbers of detections were found in Chinook Salmon (*Oncorhynchus tshawytscha*) and Coho Salmon (*O. kisutch*; USFWS 2020). However, these reports likely underestimate the true disease burden in the US, as many wild and farmed cases go unreported, untested, or undetected. Disease severity varies by fish species, age, stage of disease, and bacterial strain and ranges from an asymptomatic infection to high mortality cases (Toranzo et al. 2005). Classic BKD signs include large, swollen kidneys with grayish-white inflamed areas corresponding to granulomatous inflammation (Evenden et al. 1993). Externally, fish may appear normal or exhibit exophthalmos, coelomic distension, pale gills, hemorrhaging at the base of fins, or large abscess formation in the body cavity extending into the skeletal muscle (Bruno et al. 2013; Elliott 2017; AFS-FHS 2016).

Histopathological changes from BKD were first described by Snieszko and Griffin (1955) and Wood and Yasutake (1956) and later re-described in explicit detail as documented in the American Fisheries Society Fish Health Blue Book (Evenden et al. 1993; Elliott 2012; AFS-FHS 2016). These studies characterize BKD as a distinctive granulomatous kidney disease with macrophage infiltration and fibroblast proliferation in infected areas. Such inflammatory lesions are also associated with multifocal necrosis with various stages of encapsulation, destruction of glomeruli and tubules, and intracellular survival of *R. salmoninarum* within macrophages, causing chronic disease (Bruno 1986; Gutenberger et al. 1997; Delghandi et al. 2020). Other studies of BKD pathology describe localized lesions not only in kidney tissues, but also in the spleen, liver, heart, and brain (Wood and Yasutake 1956; Bruno 1986; Speare 1997).

Much of the knowledge regarding the pathology of BKD in salmonids comes from evaluating disease in wild populations, hatchery stocks, or experimentally infected fish to understand the post-infection immune response and does not focus on the progression of BKD throughout tissues. While studies such as Metzer et al. (2010) and Burno (1986) provide valuable insight into kidney damage from severe infections and the immune response, they focus narrowly on antigen-antibody interactions or only represent end-stage pathology observations. Understanding the sequential progression of BKD within infected fish is crucial for developing effective mitigation strategies, monitoring disease in hatcheries, and determining the appropriate tissues

and tests for detection. While *R. salmoninarum* is known to proliferate within macrophages leading to chronic granulomatous infection, the precise pathways and timing of disease dissemination among susceptible fish species remain poorly characterized.

Chinook Salmon are highly susceptible to *R. salmoninarum* infections which rapidly progress to BKD, making them a model organism to evaluate the stages of progression. To better understand disease progression in this model organism, we injected one-year-old Chinook Salmon with *R. salmoninarum* and followed the histopathology of the kidney, liver, spleen, and heart, along with bacterial load using qPCR on liver and kidney tissues, over ten weeks. Our study provides important insights into the pathology of nonfatal early to late-stage BKD, including the characteristic granulomatous nephritis that has led to diagnostic screening of kidney tissues for *R. salmoninarum*. While it is known that the infection spreads through internal tissues initiating through vertical or horizontal transmission, the timeline of disease progression is unclear. By tracking disease progression in Chinook Salmon, our observations can help determine stages of infection in affected fish and understand pathogenesis.

6.2 Methods

6.2.1 *Fish Husbandry*

Chinook Salmon (n = 70), averaging 120 ± 16 mm total length (TL) and 30.7 ± 3.5 g, were collected from the State of Idaho Pahsimeroi Fish Hatchery and transported to the Colorado State University Laboratory in Fort Collins, CO. Upon arrival, the fish were acclimated for two months to laboratory water temperature and conditions prior to the start of the experiment, after which ten fish were added to each of seven 76-L flow-through (7.5 L min^{-1}) aquaria. The Salmon were fed BioOregon feed twice a day at a standardized rate of 3% body weight based on fish size and water temperature (9.0 ± 1.4°C). Every two weeks, the total fish feed amount for each tank was adjusted by weighing all the fish and dividing by the number of fish to adjust for an increase in body weight. Feed amounts were also adjusted when a fish was removed, either from mortality or euthanization for sampling. By the end of the experiment, fish averaged 188 ± 49 mm TL and 46.6 ± 13.4 g. To maintain water quality, tanks were cleaned three times weekly with separate scrub brushes and siphons for each tank to limit cross contamination. Water hardness (150.6 $\pm$ 1.5 ppm), pH (7.6 ± 0.3), and chlorine levels (0.03 ± 0.08 mg L^{-1}), were tested biweekly using a Hach® water quality test kit, and remained consistently at those levels.

6.2.2 *Renibacterium salmoninarum Injections*

The experiment consisted of seven, 76-L tanks with ten Chinook Salmon in each tank. Five replicate tanks contained *R. salmoninarum* injected Chinook Salmon (n = 50) and two replicate control tanks (n = 20) contained Chinook Salmon injected with phosphate buffered solution (PBS) as a mock injection. Injected fish were infected with the American Type Culture Collection (ATCC) 33209D-5 isolate of *R. salmoninarum* following previously studied exposure and injection routes (Sanders and Fryer 1980; McKibben and Pascho 1999). The ATCC strain was first inoculated into kidney disease medium (KDM) broth in flasks under continuous agitation and maintained at 15°C. Every seven days for five passes, we re-cultured 1 mL of bacteria into fresh KDM broth. To verify culture purity, we performed a Gram-stain, identifying the bacterial morphology that is described for *R. salmoninarum* under 50 times magnification

(AFS-FHS 2016). The bacterial cultures were stored at -80°C prior to inoculation (OD 0.081 at 420 nm).

Chinook Salmon were anesthetized using tricaine-methanesulfonate (MS-222; 10 mg L^{-1} of water) and intraperitoneally injected with 200 µL of bacteria at an intermediate dose of 6.5 x 10^6 *R. salmoninarum* cells g^{-1} anterior to the pelvic fin (McKibben et al. 1999). Immediately after injections, fish were placed into a 5-gallon bucket with freshwater and aeration and monitored until they recovered. Once recovered, the fish were placed back into their respective experimental tanks. The same process was used for the control fish, with the exception that they were injected with 200 µL of PBS.

6.2.3 *Fish Collection*

Every week of the ten week experiment, one fish was selected from each of the experimental and control tanks and euthanized with an overdose of MS-222 to collect tissues for qPCR analysis and histopathology. The fish were monitored twice daily for signs of disease or distress that can lead to mortality. For the first six weeks, fish exhibiting the most severe external disease signs were prioritized for selection and euthanization. This included fish with symptoms such as lesions, abnormal swimming, lethargy, poor body condition, or swimming on their side. Tissues from these fish were harvested that week. These symptomatic fish would not have survived until the next sampling date and the selection process was completed to maintain fish replication in each tank throughout the experiment. However, not every tank had fish with external signs of disease, thus a random fish was sampled. Nonetheless the signs of disease observed through histology, qPCR, and Gram-stains still represent the progression of the disease. Any fish exhibiting symptoms such as lesions, abnormal swimming, lethargy, poor body condition, or swimming on its side was euthanized that week and tissues were harvested. After week six, when external disease signs were less apparent, the fish were all chosen at random for euthanization and tissue collection. Mortality was rare as a result of the selection process.

6.2.4 *Histopathology*

Immediately following euthanasia, representative sections of liver, kidney, whole spleen with surrounding adipose tissue and whole heart were removed through an abdominal excision. The kidney was split into four sections *in situ* (cranial to caudal), and the exteriorized liver was divided in two equal portions. The first (head kidney) and third (near posterior kidney) portion of the kidney, one half of the liver, and the whole spleen were used for histopathology; the remaining kidney and liver tissues were used for qPCR. The first gill arch on the left side of the fish and whole brain were also collected for histology during the dissections. All tissues used for histology were immersion fixed in 10% neutral buffered formalin for at least 24 hours. Tissue samples were trimmed and routinely processed to paraffin blocks, sectioned at seven microns, mounted on charged slides, and stained routinely with hematoxylin and eosin (H&E) for evaluation (Mondal 2017). Serial sections of all tissues were prepared routinely with Gram stain.

Hematoxylin and eosin stained liver sections were reviewed for the presence or absence of individual hepatocyte necrosis typical of sepsis, hepatic capsulitis (surface inflammation and pseudomembrane formation) indicative of coelomitis, and destructive foci of necrosis and granulomatous inflammation. Splenic tissue was evaluated for the presence or absence of

perisplenitis or coelomitis (pseudomembrane formation) and parenchymal splenitis. Kidney tissue was examined for granulomatous nephritis, tubular degeneration, and tubular necrosis. Heart tissue was evaluated for granulomatous inflammation (epicarditis, myocarditis, and endocarditis), the brain was evaluated for meningitis or encephalitis, and the gill sections were evaluated for branchitis. Gram stains were reviewed for the presence or absence of Gram-positive bacteria in each tissue. A board-certified veterinary pathologist evaluated all H&E and Gram stain preparations.

Disease progression in infected fish tissues was characterized by a stepwise pattern of pathological changes reflecting the host immune response over time. Specifically, the spleen, liver, kidney and heart each displayed a distinct sequence of one to three pathological stages as the disease advanced. Initial bacterial infection triggered an acute inflammatory response visible in the spleen as perisplenitis (stage one) - inflammation of the splenic capsule and adjacent peritoneal lining. With continued infection, inflammation extended into the spleen's parenchyma, the red pulp and lymphatic tissue composing the splenic interior as parenchymal splenitis (stage two). Similarly, the liver exhibited an incremental progression of pathology spanning three distinct stages. The first stage was individual hepatocyte necrosis consistent with sepsis (stage one). With time, inflammation spread along the hepatic capsule as hepatic capsulitis (stage two). Finally, chronic granulomatous inflammation (stage three) developed, typified by aggregations of specialized immune cells in the parenchyma. The kidney also followed a stepwise pattern, with initial interstitial nodular granulomatous nephritis (stage one), succeeded by more extensive diffuse nephritis with tubular degeneration and necrosis (stage two). The heart displayed early epicarditis, myocarditis, and/or endocarditis together as inflammation of the outer, middle, and inner heart layers. Early cases had rare individual foci of inflammation and damage, and over time, involvement of the heart tissue became more disseminated. Altogether, the host response could be characterized by one to three pathological stages reflecting the localized to widespread inflammation and tissue damage in each organ.

6.2.5 *Quantitative PCR*

Sections of liver and kidney tissue were placed into individual Whirl-Pak-bags and were frozen at -80°C upon collection until qPCR analysis. Tissues for qPCR analysis were not collected in the first week. Therefore, results only include weeks two through ten. Bacteria in liver and kidney tissues were detected and quantified following the American Fisheries Society (AFS) Fish Health Blue Book (2016) recommended testing procedures for screening for *R. salmoninarum* with qPCR. Individual tissues were thawed and homogenized in Whirl-Pak-bags using sterile rolling pins and 0.25 g of each tissue were added to sterilized microcentrifuge tubes. DNA extraction was performed with a Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) with an extra elution step to ensure collection of most of the DNA (Elliott et al. 2013). Each qPCR reaction contained 5 µL of extracted sample with the forward primer (RS 1238 5′-GTGACCAACACCCAGATATCCA-3′), the reverse primer (RS 1307 5′-TCGCCAGACCACCATTTACC-3′) and a probe with 3′ MGBNFQ quencher (RS 1262, 5′-CACCAGATGGAGCAAC-3; Chase et al. 2006). TaqMan Gene Expression Master Mix (ThermoFisher) was used at 1X concentration. An Applied Biosystems Step One Plus system was utilized with an initial incubation temperature of 50°C for two minutes, followed by 90°C for 10 min and 40 denaturing cycles at 95°C for 15 sec, followed by 60 sec of annealing at 60°C. A five-point standard curve was included in each qPCR run ranging from 1.1 x 10 to 1.1 x 10^5

bacterial cells mL^{-1} with qPCR water as a plate control (Riepe et al. *In Review*). The previously optimized qPCR assay showed a slope of the standard curve of -3.38, which corresponds to a qPCR amplification efficiency of 97.6% (MIQE standards; Bustin et al. 2009). For a sample to be considered positive for the presence of *R. salmoninarum,* the quantification cycle (Cq) value had to be less than 37.75 corresponding to an analytical sensitivity of 1.1 bacterial cells mL^{-1}. (Sandell and Jacobson 2011; Riepe et al. *In Review*). The number of bacteria in each sample was estimated by comparing the Cq values for the positive tissue samples to the previously developed standard curve, and then converted to the number of bacteria per gram of tissue (bacteria g^{-1}).

6.2.6 *Statistical Analysis*

For kidney and liver tissues, linear models with an analysis of variance (ANOVA) were used to examine relationships between disease progression over ten weeks and bacterial load (number of bacteria g^{-1} of tissue). The same approach was used to examine the relationship between disease stages and bacterial load. Specifically, the $\log_{10}$ bacterial load was regressed against the interaction of disease stage and tissue type to assess significance.

Detection of parasites occurred with histology throughout the experiment and was unexpected. To evaluate if the presence of the parasites affected BKD progression, we used a linear mixed effects model with disease stage from histology as the response variable, and parasite presence and parasite tissue location (liver or kidney) as predictor variables. Individual fish were included as a random intercept to account for repeated, non-independent parasite observations per fish (two tissues from each of 42 *R. salmoninarum*-injected fish; n = 84 observations). All analyses were performed in Rstudio version 4.1.0 and significance was set at 0.05 (α).

6.3 Results

Chinook Salmon injected intraperitoneally with *R. salmoninarum* developed postmortem disease signs within 21 days. Postmortem signs of disease began with pale coloration of liver tissue appearing in week three, followed by granulated spleen and white kidney spots in week four. Later signs included lethargy in week six and ascites in week seven. Disease signs accumulated over the ten-week study, with individual fish exhibiting multiple signs in later weeks. Mortality began in week seven, with three total mortalities. Control fish showed no signs of disease, lesions, or mortality.

6.3.1 *Histopathology*

The control fish showed no evidence of hepatic capsulitis, parenchymal hepatitis, perisplenitis, or splenitis, pathologies typically associated with BKD. However, mild hepatic necrosis occurred in a few control fish (one in weeks four and six, and two fish in week nine). Additionally, mild epicarditis was observed in two control fish (one in week six and one in week nine), and mild to moderate proliferative branchitis was present in all control fish and *R. salmoninarum* injected fish. The results below focus on the ten-week progression of disease caused by *R. salmoninarum* infection, specifically in the spleen, liver, kidney, heart, and gill tissues.

Disease progression was categorized into one to three stages for the liver, spleen, and kidney tissues, including signs of acute disease after initial bacterial injection and the infected tissue's response over time (Figure 6.1). Once organs were observed in the next stage of infection, the

proportion of fish showing that stage of infection increased into the later weeks of the experiment (Table 6.1).This staged pattern gives insight into the time course and progression of disease in different tissues following initial bacterial infection and is described below.

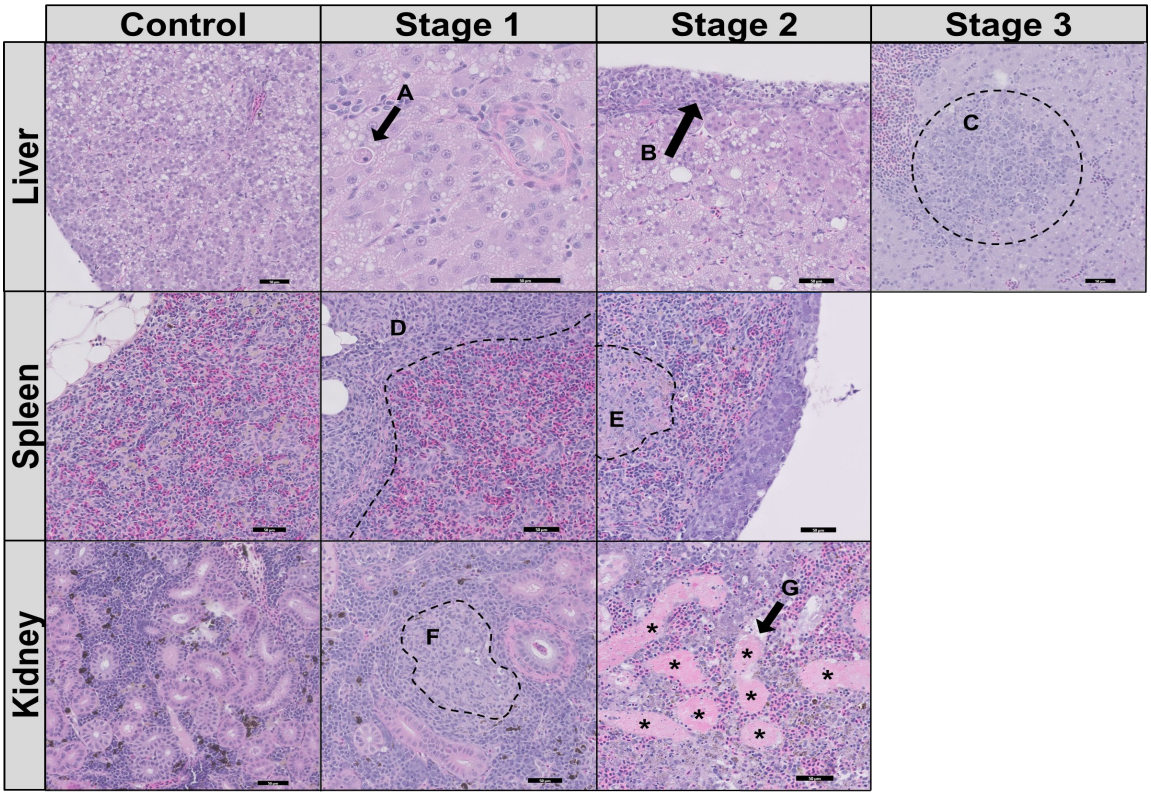


Figure 6.1 Histopathological sections of *Oncorhynchus tshawytscha* experimentally infected with *Renibacterium salmoninarum* showing two to three stages of disease progression. Hepatic (liver) tissue first showed individual hepatocyte necrosis; necrotic hepatocytes are shrunken and hypereosinophilic with a pyknotic nucleus (A), then granulomatous capsulitis (B), and granulomatous parenchymal inflammation (C). Splenic (spleen) tissue first showed granulomatous perisplenitis (D; left of dotted line) and progressed to granulomatous parenchymal splenitis (E; outlined) with concurrent perisplenitis. Renal (kidney) tissue first showed multifocal granulomatous nephritis (F) and progressively severe coalescent granulomatous nephritis with tubular degeneration and acute necrosis (G); necrotic tubules are marked by asterisks. Histopathological sections from control fish are included as a reference. Scale bars represent 50 µm for all images.

Histopathological changes in the tissues caused by *R. salmoninarum* were similar to the lesions identified in previous studies (Bruno 1986; Speare 1997; AFS 2016). In weeks one and two, the fish had random individual hepatocyte necrosis in the liver, supportive of sepsis (stage one). Rare and scant hepatic capsulitis (pseudomembrane formation; stage two) began in week three and was more consistently present in fish in week four. This was characterized by an accumulation of bacteria-laden macrophages, lymphocytes, fibrin, and reactive fibroblasts at the capsular surface. The hepatic capsulitis progressed to intrahepatic granulomatous hepatitis (stage three) from weeks seven through ten (Table 6.1).

Table 6.1 Histopathology detection for *Renibacterium salmoninarum* in liver, spleen, kidney and heart tissues. Proportions represent detection of each stage of disease progression for tissues out of five total fish collected in weeks one through nine and two fish collected in week ten. Dashes indicate no detection of *Renibacterium salmoninarum*.

	Liver			**Spleen**		**Kidney**		**Heart**
	Stage 1	**Stage 2**	**Stage 3**	**Stage 1**	**Stage 2**	**Stage 1**	**Stage 2**	**Stage 1**
	Necrosis	Capsulitis	Granulomatous Inflammation	Perisplenitis	Parenchymal Splenitis	Nephritis	Nephritis w/Necrosis	Epicarditis
Week 1	0.6	-	-	0.6	-	-	-	-
Week 2	1	-	-	0.6	-	-	-	0.2
Week 3	0.4	0.2	-	1	-	-	-	0.2
Week 4	1	0.6	-	1	-	-	-	0.4
Week 5	0.6	0.4	-	1	-	0.8	-	0.6
Week 6	0.4	0.2	-	0.8	-	0.4	-	0.4
Week 7	1	0.6	0.2	0.8	0.4	0.4	0.2	0.4
Week 8	1	0.6	0.6	1	0.4	0.8	0.4	0.4
Week 9	1	0.8	0.6	1	0.4	0.8	-	0.8
Week 10	1	1	1	1	0.5	1	-	0.5

The spleen showed prominent granulomatous perisplenitis in the first week (stage 1) and progressed over time. Histological examination revealed accumulation of bacteria-laden macrophages, lymphocytes, and fibrin, with florid bacteria detected beyond week four. Perisplenic infiltrates frequently extended several millimeters through local coelomic adipose tissue. By weeks seven through ten, necrotizing and granulomatous splenitis as well as perisplenic steatitis were prominent (stage 2; Table 6.1).

The kidney tissue in week five showed focal to multifocal granulomatous nephritis histologically (stage 1). Initially, macrophages containing bacteria formed small nodular aggregates (typically less than 500 microns in diameter) that displaced tubules and glomeruli. By weeks seven through ten, nephritis had markedly progressed with diffuse interstitial infiltration by numerous macrophages and both intra- and extracellular bacteria. There was also multifocal acute tubular degeneration and necrosis (stage 2). Necrotic tubules were hypereosinophilic with pyknotic or absent nuclei. In some areas, coagulative necrosis affected large parenchymal regions. Occasional acute emboli of bacteria-laden macrophages were trapped in glomerular capillaries, although chronic glomerulonephritis was not observed (Table 6.1).

In weeks seven through ten, the number of fish with granulomatous epicarditis, myocarditis, and endocarditis progressively increased. Intravascular circulating macrophages with Gram-positive bacteria were observed in the heart and the gill in week seven (Figure 6.2) and in the liver in week 9. Fish from weeks seven to ten had very high burdens of *R. salmoninarum* and greater tissue damage in the spleen, liver, and kidney tissue as well as in the heart, with many fish showing terminal bacteremia. Although *R. salmoninarum* has been shown to cause encephalitis and meningitis in Atlantic Salmon and Chinook Salmon (Speare 1997), we did not detect bacteria or histological signs of brain disease over the ten-week experiment.

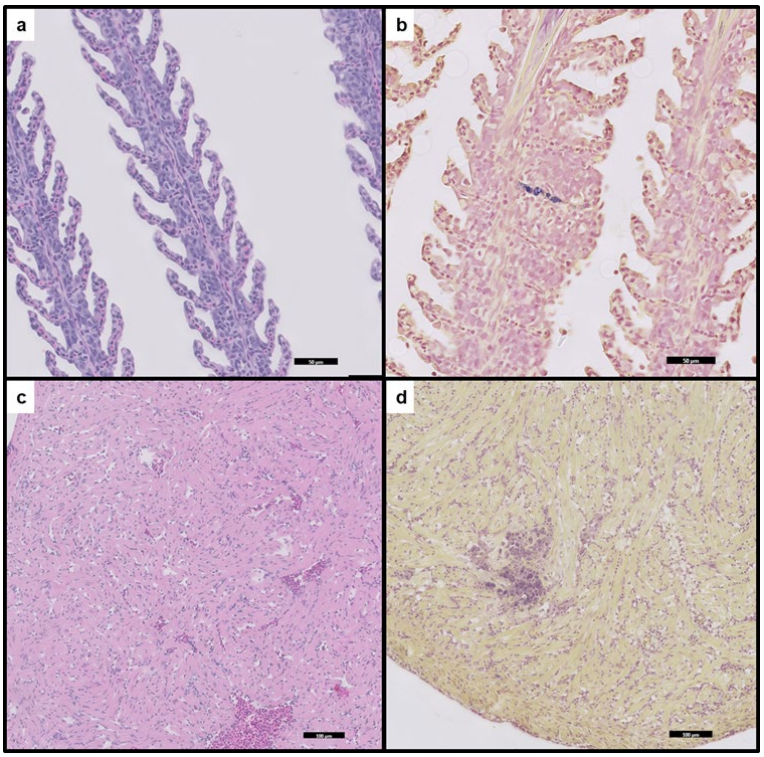


Figure 6.2 H&E of gills from a control fish showing modest branchitis typical of that seen in fish from the study (a), and Gram stained section from an experimentally injected fish in week 8 (b) with Gram-positive bacterial thrombi in secondary gill lamellae. H&E cardiac tissue from a control fish (c), and Gram stained section of heart from an experimentally injected fish at week 10 with myocarditis with Gram-positive bacteria (d). Scale bars represent 50 µm for gills and 100 µm for cardiac tissue.

Suspected sanguinicolid fluke eggs were observed in the kidney and less frequently in the myocardium or branchial interstitium. The metazoan eggs were sometimes associated with localized granulomatous inflammation, distinct from the more severe nodular to diffuse granulomatous inflammation induced by *R. salmoninarum* infection (Figure 6.3a). Unidentified myxozoan parasite life stages were sometimes present within the kidney tubular epithelium and led to epithelial hyperplasia without significant inflammation (Figure 6.3a). Parasite presence did not significantly impact BKD progression in the kidney, liver, or spleen tissue (t = -0.68, p-value = 0.25).

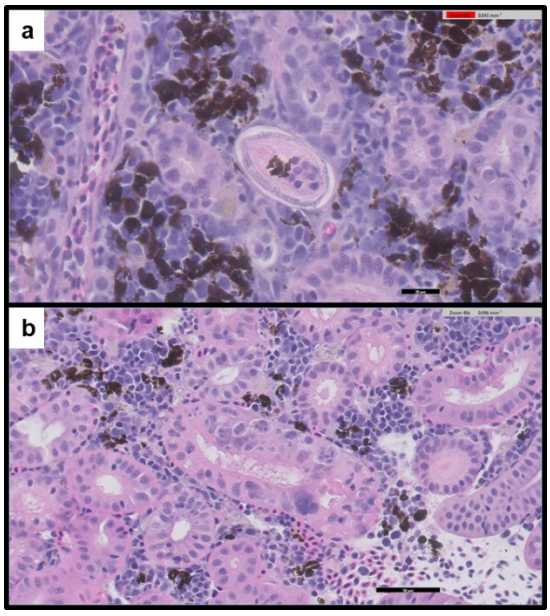


Figure 6.3 Incidental histopathology findings of (a) metazoan eggs (Scale – 20 Lm) and (B) intraepithelial myxozoans in renal tubular epithelium (scale = 50 µm), H&E preparation.

4.3.2 *Gram Stain*

Gram-positive bacteria were not observed in any control fish. In *R. salmoninarum* injected fish, Gram stains showed Gram-positive bacteria within macrophages first in the perisplenic adipose tissue. In later weeks, Gram-positive bacteria appeared in granulomatous inflammation in the liver, kidney, and spleen tissue (Table 6.2; Figure 6.4). Gram-positive bacterial thrombi were sometimes visible in glomerular capillaries and secondary gill lamellae (Figure 6.2). From weeks seven through ten, Gram-positive bacteria were evident in sites of heart inflammation including epicarditis, myocarditis, and endocarditis. In terminal fish, Gram-positive bacteria were observed inside macrophages in the bloodstream.

Table 6.4 Gram-positive tissues are represented across the ten-week experiment as a percentage of fish positive out of five total fish collected in weeks one through nine and two fish collected in week ten. Dashes indicate no detection of *Renibacterium salmoninarum*.

	Spleen	**Liver**	**Kidney**	**Heart**	**Gill**
Week 1	0.4	-	-	-	-
Week 2	0.2	-	-	-	-
Week 3	0.6	-	-	-	-
Week 4	1	0.2	-	-	-
Week 5	1	0.6	0.4	-	-
Week 6	0.4	0.2	0.2	-	-
Week 7	0.6	0.2	0.4	0.2	0.2
Week 8	1	0.6	0.8	0.4	0.2
Week 9	1	0.6	0.8	0.4	0.4
Week 10	1	1	0.5	0.5	0.5

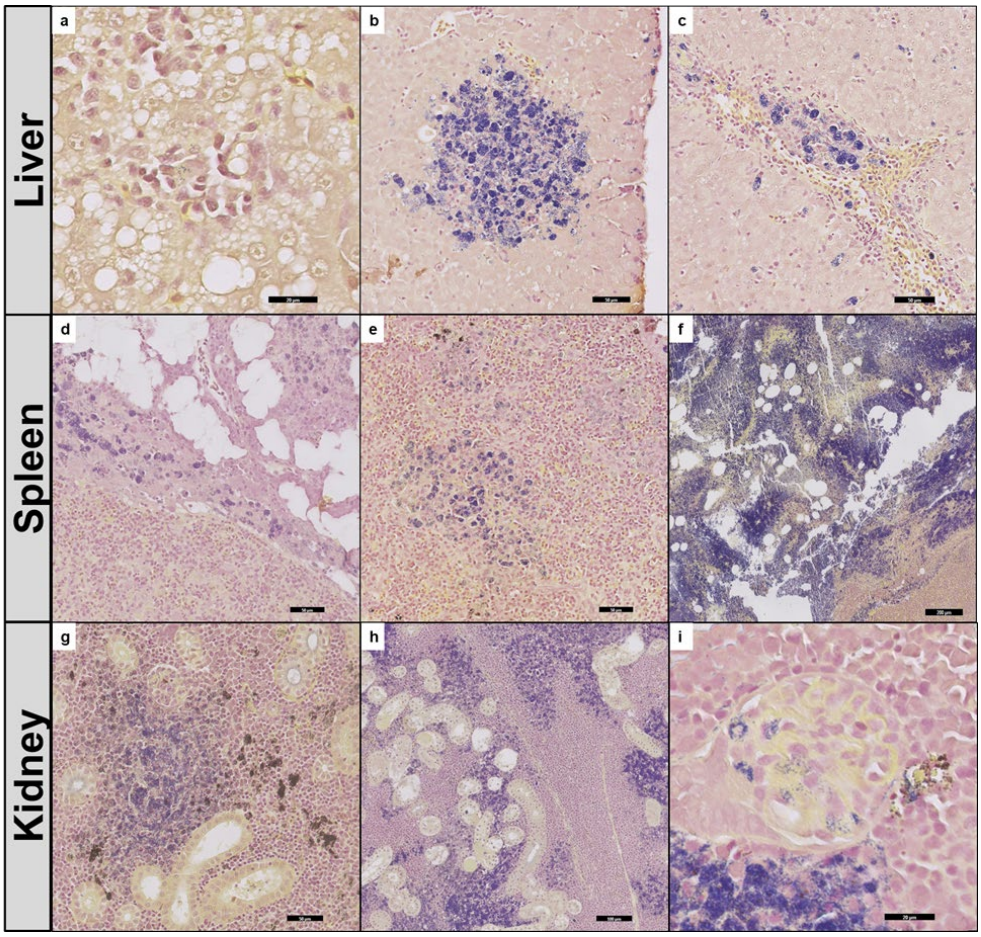


Figure 6.4 Gram stain of liver, kidney and spleen. (a) Early signs of *Renibacterium salmoninarum* presence in a week 4 liver sample with rare Gram-positive bacteria (scale = 20 Lm). (b) Hepatitis with Gram-positive bacteria in a week 8 liver sample (scale = 50 µm). (c) Hepatitis and bacteria in sinusoidal macrophages in week 8 liver sample (scale = 50 µm). (d) Early signs of *R. salmoninarum* presence in week 9 spleen sample (scale = 50 µm). (e) Splenitis in week 7 spleen sample (scale = 50µm). (f) Perisplenic peritonitis in week 10 spleen sample (Scale = 100 µm). (g) Early aggregate of macrophages containing *R. salmoninarum* in week 10 kidney sample (scale = 50 µm). (h) Severe nephritis in a week 10 kidney sample (scale = 100 µm). (i) Glomerular emboli in week 8 kidney sample (scale = 20 µm)

Prior to detecting Gram-positive bacteria in week four, individual hepatocyte necrosis and capsulitis were observed in the liver. Similar patterns occurred in heart tissue, with inflammation seen in week two but no Gram-positive bacteria detected until week seven (Figure 6.2). In contrast, histological and Gram-positive bacteria were detected concurrently in perisplenic tissues and kidney in weeks one and five, respectively.

6.3.3 *Quantitative PCR*

Quantitative PCR revealed low levels of *R. salmoninarum* infection in control fish, averaging 69.37 ± 156.79 bacteria g^{-1} in kidney tissues and 24.95 ± 72.66 bacteria g^{-1} in liver tissue. This was expected as the fish came from a previously known positive hatchery for *R. salmoninarum*. Since these bacteria levels were so low, we are not concerned with our overall results from the infection study. Over the ten week experiment, bacterial loads ranged from 5.30 to 1,632,151.81 g^{-1} kidney tissue and from 0.82 to 1,116,513.60 bacteria g^{-1} liver tissue. Bacterial loads in both

kidney and liver tissues increased significantly throughout the experiment (Kidney $F_{8,36} = 2.69$, p-value < 0.05; Liver $F_{8,36} = 3.45$, p-value < 0.001; Figure 6.5).

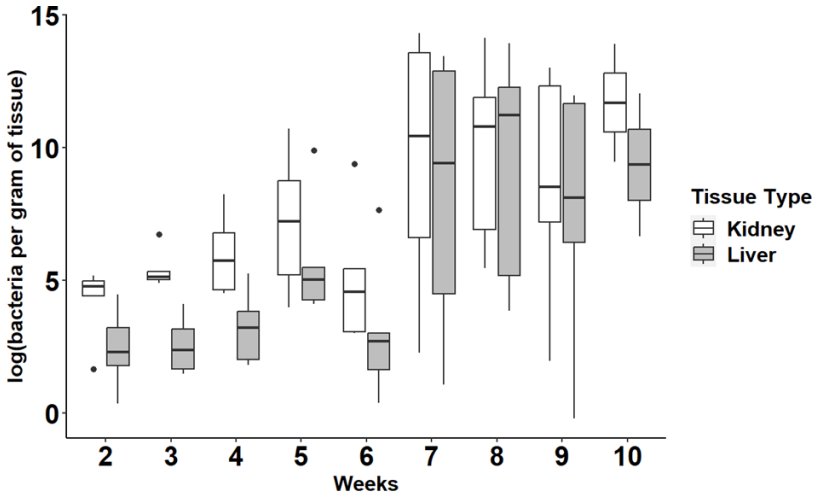


Figure 6.5 The number of *Renibacterium salmoninarum* bacteria per gram of tissue on a log scale across weeks two through ten for kidney (white) and liver tissues (gray). Each tissue and week represents log(bacteria per gram of tissue) for five injected fish each. Boxplot extent ranges represent the 25th and 75th percentile; band near the middle of the box represents the 50th percentile/median; whiskers range from lowest to highest with points as outliers.

Kidney tissues contained higher bacterial loads than liver tissues across all ten weeks, except week eight where liver samples held higher bacterial averages. Interestingly, both tissues showed a decline in bacterial load at week six, possibly due to our randomized fish selection as fish were no longer showing external signs of disease after week five. As expected, disease progression stage strongly predicted log(bacteria g^{-1} tissue) in a linear fashion for both kidney and liver samples in experimental fish (stage $F_{3,77} = 13.60$, p-value < 0.001; stage x tissue $F_{2,77} = 3.31$, p-value < 0.05; Figure 6.6).

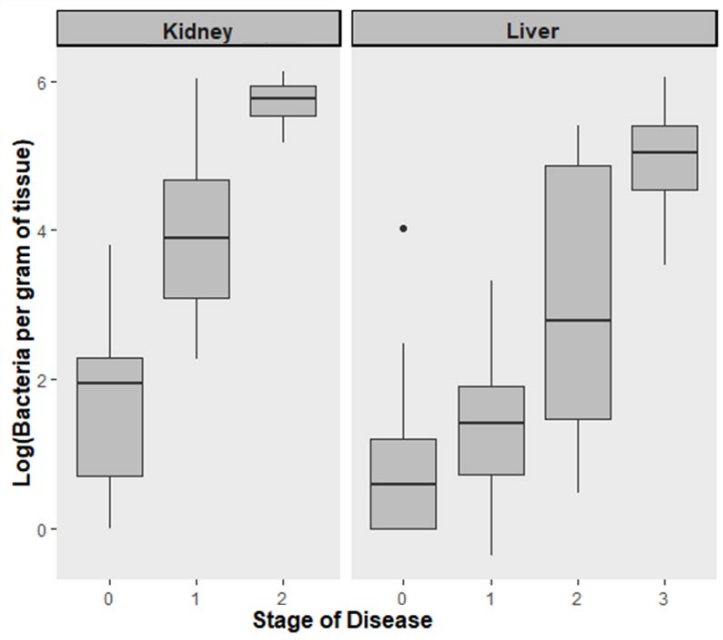


Figure 6.6 The log(bacteria per gram of tissue) as a function of histological stage of disease for the kidney and liver tissues test from week two through week ten. Histologic stages of disease for each tissue represent the following: Kidney – no disease (stage 0), nephritis (stage 1), and nephritis with necrosis (stage 2). Liver – no disease (stage 0), hepatic necrosis (stage 1), hepatic capsulitis (stage 2), and granulomatous inflammation (stage 3).

6.4 Discussion

Our study contributes to a growing body of literature indicating that, despite its name, bacterial kidney disease is not limited to the kidney, and may be more common in other organs depending on when exposure to the bacteria occurred and the progression of the disease throughout tissues. Our results indicate that after intraperitoneal injection in Chinook Salmon, *R. salmoninarum* infection progressed from the spleen to the liver, and then kidney. We did not detect the bacteria or histological disease signs in kidney tissues until week five post-injection even though the fish that showed signs of disease were sampled first. However, bacteria and disease signs appeared in the spleen and liver as early as week one. Current screening recommendations for *R. salmoninarum* do not include the spleen and liver, but our results suggest these organs may be used for early detection shortly after a suspected exposure and even weeks later, since the bacteria remain in tissues throughout disease progression.

Bacterial kidney disease is a progressive, multi-systemic infection that spreads beyond the kidney to infect other organs. Our findings confirm previous studies showing that BKD affects not just the kidney, but also the liver (Kent et al. 2013; Delghandi et al. 2020), spleen (Flaño et al. 1996a; Kent et al. 2013; Delghandi et al. 2020), heart (Wood and Yasutake 1956; Bruno 1986), and gill tissues (Flaño et al. 1996b; Bruno et al. 2013). Additionally, our study suggests the spleen and liver are infected prior to the kidney tissue. However, Metzger et al. (2010) exposed Chinook Salmon intraperitoneally and found that kidney tissues are infected first followed by spleen and liver tissue as observed with histological detection. After two weeks post-infection, they found that 80% of the fish showed histological disease in kidney tissues, whereas only 40% showed signs in the liver and 60% in the spleen, despite using similar

injection doses to our study. Additionally, the fish showed high IFN-γ gene expression in kidney tissues, which indicates cell mediated immune response. It is surprising to us that Metzger et al. (2010) showed more frequent histological involvement of the kidney than the spleen since cell mediated immunity following bacterial infection occurs in the spleen first (Ashfaq et al. 2019). Nonetheless, we are confident in our results, in which we detected Gram-positive bacteria in the spleen and liver prior to the kidney.

The stages of progression for kidney and liver tissue compared to qPCR results indicate there is a relationship between qPCR infection load and histologic progression that we defined in our study. Liver granulomas have previously been observed via histology and confirmed *R. salmoninarum* infections, even when fish were negative by ELISA or direct fluorescent antibody tests (DFAT) and in the absence of granulomas in the kidney and spleen (DFAT; Kent et al. 2013). The comparison in detection rates between histology, Gram stains and qPCR highlight the importance of using multiple testing methods for *R. salmoninarum* detection. Quantitative PCR was more sensitive than histopathology at detecting infection, even when histopathology was paired with Gram stains. For instance, qPCR detected low levels of bacterial DNA in control tissues when no lesional tissue or recognizable disease was present histologically. This likely occurred because qPCR primers have high affinity for their targets and the process amplifies even very low copy numbers of DNA. As a result, qPCR is ideal for detecting early or subclinical disease. In contrast, histopathology can miss lesional areas if they are rare or the histologic areas of disease are not represented. Thus, histopathology may underestimate infection in its early stages or in tissues that are not primary infection sites. In our study, histopathology likely missed some early myocarditis, pseudomembrane formation, inflammation, or asymmetric lesions not represented in the cross-sectioned samples. However, in the liver, spleen and kidney tissues, histopathologic evidence of *R. salmoninarum* infection was usually rapid, unequivocal, and multifocal to diffuse once bacterial burden increased. Despite low sensitivity with low bacterial infections, histopathology was critical for detecting progression over time and specific features of damage such as inflammation and necrosis. It also allowed for detection of incidental parasites.

Current diagnostic testing methods suggest collecting kidney tissues for the detection of *R. salmoninarum* (AFS 2016); however, new findings suggest that liver tissue should be used for diagnostic tests (Riepe 2022; Riepe et al. 2023). In Riepe et al. *In Review*, the probability of detecting bacteria by qPCR *R. salmoninarum* infected fish was 57.2% in liver tissue and only 32.7% in kidney tissue. While the authors of that study did not understand why bacterial detection was greatest in the liver tissues at the time, these new results indicate that with IP exposure, bacteria may infect the liver prior to the kidney. This was observed in both the histology and Gram-positive detections which occurred in the liver prior to the kidney. For instance, histology showed perisplenic inflammation with Gram-positive bacteria and individual hepatocyte necrosis consistent with sepsis as early as one week post-infection, while kidney inflammation was not detected until week five. Early Gram-positive detection in the spleen (week 1) and liver (as early as week 3) supports these findings. Thus, the inclusion of liver as well as kidney for detection of *R. salmoninarum* in the early stages of infection may offer increased likelihood of detection.

The results of this ten week study suggest an increase in bacterial loads and histological signs of infection in tissues over time. However, our data indicate a decrease at week six. The decline

may be attributed to preferentially selecting the most clinically-sick fish for sampling until week six. Once those fish were removed, the remaining, generally healthier fish likely had lower bacterial loads and fewer histological signs of infection. A limitation of our study is the lack of splenic qPCR data, as we did not expect spleen involvement initially. Future work should evaluate splenic bacterial loads for comparison. However, our histology results suggest that spleen is an ideal candidate tissue for *R. salmoninarum* detection. Additionally, exposure routes may affect disease progression and tissue involvement. Though injections do not mimic natural exposure, they standardize the initial dose and allow us to compare across other studies. Further studies should mimic a natural infection route via horizontal transmission with a similar approach to follow disease progression throughout tissues.

6.5 References

AFS-FHS (American Fisheries Society-Fish Health Section). 2016. FHS Blue Book: Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2020th ed.

Ashfaq, H., H. Soliman, M. Saleh, and M. El-Matbouli. 2019. CD4: A vital player in the teleost fish immune system. Veterinary Research 50:1.

Bruno, D. W. 1986. Histopathology of bacterial kidney disease in laboratory infected Rainbow Trout, *Salmo gairdneri* Richardson, and Atlantic Salmon, *Salmo salar* L. with reference to naturally infected fish. Journal of Fish Diseases 9: 523-537.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, and J. Vandesompele. 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry 55(4): 611□622.

Bruno, D. W., P. A. Noguera, and T. T. Poppe. 2013. Bacterial diseases. *In* A colour atlas of salmonid diseases (2nd ed.). Springer, Berlin, Heidelberg.

Causey, D. R., M. A. N. Pohl, D. A. Stead, S. A. M. Martin, C. J. Secombes, and D. J. Macqueen. 2018. High-throughput proteomic profiling of the fish liver following bacterial infection. BMC Genomics 19: 719.

Chase, D. M., D. G. Elliott, and R. J. Pascho. 2006. Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. Journal of Veterinary Diagnostic Investigation 18(4): 375-380.

Delghandi, M. R., M. El-Matbouli, and S. Menanteau-Ledouble. 2020. *Renibacterium salmoninarum*—The causative agent of bacterial kidney disease in salmonid fish. *Pathogens* 9(10):845.

Elliott, D. G. 2012. Bacterial kidney disease. *In*: AFS-FHS (American Fisheries Society-Fish Health Section). FHS Blue Book: Suggested procedures for the detection and

identification of certain finfish and shellfish pathogens, 2016 edition. AFS-FHS, Bethesda, Maryland.

Elliott, D. G., L. J. Applegate, A. L. Murray, M. K. Purcell, and C. L. McKibben. 2013. Bench-top validation testing of selected immunological and molecular *Renibacterium salmoninarum* diagnostic assays by comparison with quantitative bacteriological culture. Journal of Fish Diseases 36(9), 779-809.

Elliott, D. G. 2017. *Renibacterium salmoninarum*. *In*: Fish viruses and bacteria: Pathobiology and protection, pp. 288-297. Wallingford UK: CABI, 2017.

Evelyn, T. P. T., J. E. Ketcheson, and L. Prosperi-Porta. 1986. Use of erythromycin as a means of preventing vertical transmission of Renibacterium salmoninarum. Diseases of Aquatic Organisms, 2(1), 7-11.

Evenden, A. J., T. H. Grayson, M. L. Gilpin, and C. B. Munn. 1993. *Renibacterium salmoninarum* and bacterial kidney disease — the unfinished jigsaw. Annual Review of Fish Diseases 3:87–104.

Flaño, E., P. López-Fierro, B. Razquin, S. L. Kaattari, and A. Villena. 1996a. Histopathology of the renal and splenic hematopoietic tissues of Coho Salmon *Oncorhynchus kisutch* experimentally infected with *Renibacterium salmoninarum*. Diseases of Aquatic Organisms 24:107-115.

Flaño, E., P. López-Fierro, B. Razquin, and A. Villena. 1996b. *In vitro* differentiation of eosinophilic granular cells in *Renibacterium salmoninarum*-infected gill cultures from Rainbow Trout. Fish and Shellfish Immunology 6(3):173–184.

Griffiths, S. G., K. J. Melville, and K. Salonius. 1998. Reduction of *Renibacterium salmoninarum* culture activity in Atlantic Salmon following vaccination with avirulent strains. Fish and Shellfish Immunology 8(8):607–619.

Gudmundsdóttir, S., L. J. Applegate, Í. Ö. ÁrnasonÍ, Á Kristmundsson, M. K. Purcell, and D. G. Elliott. 2017. Detecting *Renibacterium salmoninarum* in wild Brown Trout by use of multiple organ samples and diagnostic methods. Bulletin of the European Association of Fish Pathologists 37:31-40.

Kent, M. L., S. Benda, S. St-Hilaire, and C. B. Schreck. 2013. Sensitivity and specificity of histology for diagnoses of four common pathogens and detection of nontarget pathogens in adult Chinook Salmon (*Oncorhynchus tshawytscha*) in fresh water. Journal of Veterinary Diagnostic Investigation 25(3):341-351.

McKibben, C. L. and R. J. Pascho. 1999. Shedding of *Renibacterium salmoninarum* by infected Chinook Salmon *Oncorhynchus tshawytscha*. Diseases of Aquatic Organisms 38: 75–79.

Metzger, D. C., D. G. Elliott, A. Wargo, L. K. Park, and M. K. Purcell. 2010. Pathological and immunological responses associated with differential survival of Chinook Salmon

following *Renibacterium salmoninarum* challenge. Diseases of Aquatic Organisms 90(1): 31-41.

Mondal, S. K. 2017. Manual of histological techniques. JP Medical Ltd.

Riepe, T. B. 2022. Transmission and detection of *Renibacterium salmoninarum* in Colorado inland trout. Colorado State University, Ph.D. Dissertation.

Riepe, T. B., E. R. Fetherman, B. Neuschwanger, T. Davis, A. Perkins, and D. L. Winkelman. 2023. Vertical transmission of *Renibacterium salmoninarum* in Cutthroat Trout (*Onchorhynchus clarkii*). Journal of Fish Diseases 46:309–319.

Sandell, T. A. and K. Jacobson. 2011. Comparison and evaluation of *Renibacterium salmoninarum* quantitative PCR diagnostic assays using field samples of Chinook and Coho Salmon. Diseases of Aquatic Organisms 93:129–139

Sanders, J., and J. L. Fryer. 1980. *Renibacterium salmoninarum* the causative agent of bacterial kidney disease in salmonid fishes. International Journal of Systematic and Evolutionary Microbiology 30: 496–502.

Snieszko, S. F. and P. J. Griffin. 1955. Kidney disease in Brook Trout and its treatment. The Progressive Fish-Culturist 17(1): 3–13.

Speare, D. J. 1997. Differences in patterns of meningoencephalitis due to bacterial kidney disease in farmed Atlantic and Chinook Salmon. Research in Veterinary Science 62: 79–80.

Toranzo, A. E., B. Magarinos, and J. L. Romalde. 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture 246: 37–61.

Wood, E. M. and T. Yasutake. 1956. Histopathology of kidney disease in fish. American Journal of Pathology 32:845–857.

USFWS. 2020. U.S. Fish and Wildlife Service: National Wild Fish Health Survey Data. Accessed from: https://www.fws.gov/project/national-wild-fish-health-survey-data.

Thriving in Extreme Environments: Salinity and Thermal Tolerance of the Western Mosquitofish (*Gambusia affinis*) and Plains Topminnow (*Fundulus sciadiscus*)

Samuel Lewis (Ph.D. Candidate), Dr. Paula A. Schaffer, Dr. Pete Cadmus

7.1 Introduction

Invasive species pose a serious threat to global biodiversity, often outcompeting native species and altering ecosystem dynamics (Lennox et al. 2015). Freshwater ecosystems are uniquely susceptible to invaders (Strayer 2010) due to widespread habitat degradation and simplification, which favor generalists (Dudgeon et al. 2006; Moorhouse and MacDonald, 2015). As such, freshwater ecosystems have suffered from introductions of non-native species causing declines in global aquatic species richness (Miller et al. 1989; Dias et al. 2017). The impact of introduced species extends beyond ecological harm having cost the global economy a total of 345 billion US dollars in mitigation efforts (Cuthbert et al. 2021). The mechanisms driving these invasions are diverse, including predation, hybridization, and disease transmission (Berthou 2007). One leading hypothesis is that invasive species may possess greater tolerance to changing abiotic conditions and environmental degradation giving them a competitive edge in disturbed habitats (Alcaraz et al. 2008; Kelley, 2013). Understanding the abiotic factors that contribute to the success of invasive species is therefore crucial for developing effective management and conservation strategies for native species.

The ability of introduced species to thrive in new environments is often linked to their physiological tolerance of a wide range of abiotic conditions. The potential for invasive species to replace native species due to differing physiological tolerances, particularly in the face of environmental change, has been a focal point in invasion ecology (Kelley 2014; Gessner and Tlili 2016). This tolerance, coupled with life-history traits like early maturation and high fecundity, can facilitate rapid proliferation and displacement of native species (Lee et al. 2003). The invasion success of species like the Western Mosquitofish (*Gambusia affinis*) exemplifies this phenomenon. Western Mosquitofish are listed on the International Union for Conservation of Nature's (IUCNs) 100 worst invasive alien species having been documented on every continent except Antarctica (Pyke 2008). These fish exhibit a remarkable capacity to withstand diverse and often harsh environmental conditions (Lee et al. 2003 Laha and Mattingly 2006). This adaptability stems from their physiological plasticity, allowing them to adjust their internal mechanisms in response to external stressors like fluctuating temperature and salinity (Reeve 2014; Santi et al. 2020; Zhou et al. 2022). This capacity to tolerate a broader range of abiotic conditions than their native counterparts provides them with a critical advantage in disturbed or changing environments (Alcaraz et al. 2008). Notably, even subtle behavioral changes in response to sublethal exposure to contaminants can have cascading effects on species interactions, potentially benefiting more tolerant invasive species (Fleeger et al. 2020; Desforges et al. 2023)

Western Mosquitofish have been documented in Colorado's South Platte River basin since the 1990's and have seen great expansions in their range encroaching on critical habitat for many native eastern plains fish (Colorado Parks and Wildlife 2015). The eastern plains of Colorado

present a challenging environment for many aquatic species. Extreme temperature fluctuations, salinity, periodic drying, and long-term drought have degraded many aquatic communities in the region (Dodds et al. 2004; Falke et al, 2011). Despite these challenges, the South Platte and Republican River basins support a surprising diversity of fish species. Thirty-six native fish species are found in these basins, with twelve classified as Species of Greatest Conservation Need (SGCN) by the state of Colorado (Colorado Parks and Wildlife 2015). The Mosquitofish's expansion and furthered invasion is particularly concerning given the already precarious state of many native fish populations in the basin.

Water management practices in the South Platte River basin further complicates the coexistence between the introduced Western Mosquitofish and other native fish species. The South Platte is often described as a "working river" as by the time it reaches the Nebraska/Kansas border water has been re-used at least six times (Strange et al. 1999). This practice is especially common in the agriculturally dominated eastern plains, where water is diverted for irrigation and returned as irrigation return flows (SBIP 2022). These return flows, while sustaining downstream flow, carry a significant load of dissolved solids from the soil, leading to increased salinity downstream (O'Brien 2020; Hosseini and Bailey 2022). This increase in salinity exposes aquatic organisms to increasingly saline conditions creating key avenues for invasive species to survive in instances of poor water quality.

The harsh environmental conditions of prairie ecosystems are further characterized by extreme temperature fluctuations and a scarcity of thermal refugia (Dodds et al. 2004; Hopper et al. 2020). The South Platte River basin experiences significant fluctuations in water temperature and flow, particularly in the lower reaches dominated by agricultural land use (Sprague 2005; SBIP 2022). These areas, often characterized by shallow, slow-moving water, are susceptible to dramatic temperature swings, exceeding the thermal limits of many native fish species (Hopper et al. 2020). The ability of Western Mosquitofish to tolerate such extreme temperature fluctuations, combined with their capacity to thrive in degraded water conditions, might give them a significant advantage in these habitats. This advantage, coupled with their high reproductive rate and broad diet, could allow Western Mosquitofish to outcompete native species for resources and further establish themselves within these already stressed ecosystems. Notably, studies on other fish species have shown that those inhabiting intermittent streams, characterized by periodic drying, often face lethal temperatures as water levels recede and temperatures rise, highlighting the vulnerability of native species in these habitats (Dodds et al. 2004; Hopper et al. 2020). The resilience of Western Mosquitofish in the face of such challenging thermal conditions underscores their potential to thrive in prairie stream ecosystems and potentially displace less tolerant native species.

This study examined tolerances of the invasive Western Mosquitofish relative to a native competitor, the Plains Topminnow (*Fundulus sciadicus*). Laboratory toxicity trials were employed to (1) compare the salinity tolerances between the two species, (2) determine the respective critical thermal maxima (CTM) of each species, and (3) identify possible synergistic effects between increased salinity and increased temperature. We predicted that as a prolific invader Western Mosquitofish's tolerance to the abiotic factors of salinity and temperature could

explain some of the ability to thrive in Colorado's eastern plains streams, even during extreme events.

7.2 Methods

7.2.1 *Data Collection*

Mosquitofish were collected from the Running Deer Natural Area, located near Colorado State University in Fort Collins. Using dip nets, fish were gathered from streams where Mosquitofish coexist with Plains Topminnow, although they significantly outnumber the native species. Plains Topminnow were collected from the Pawnee National Grasslands from small pools where they thrive in large numbers in the absence of Mosquitofish. Both field sites were characterized by large pond complexes in the floodplain areas with dense submerged aquatic vegetation. Fish were transported in oxygenated and insulated coolers to holding tanks at the Colorado Parks and Wildlife Aquatic Toxicology Laboratory (Fort Collins, CO, USA). Fish were immersed in a 37% formaldehyde solution for 1 hour to prophylactically treat any potential parasites. For over 48 hours fish were acclimated in 500 L holding tanks receiving flow through dechlorinated municipal tap water help at 20ºC. (Figure 7.1C Pink insulated aquaria). Forty eight hours prior to toxicant exposure fish were randomly assigned to 48 aquaria (Figure 7.1B) in water baths (Figure 7.1C). Aquaria were fed 40 mL/min of dechlorinated municipal tap water downstream of a gravity fed serial dilution system based on Benoit (1985). Chilling and heating systems held flow-through water and water baths at 20ºC. After a >24-hour acclimation toxicant was introduced and salinity levels began to increase over the next 24 hours until stabilizing at six experimental concentrations (2400 mg/L, 1200 mg/L, 600 mg/L, 300 mg/L, 150 mg/L and a control).

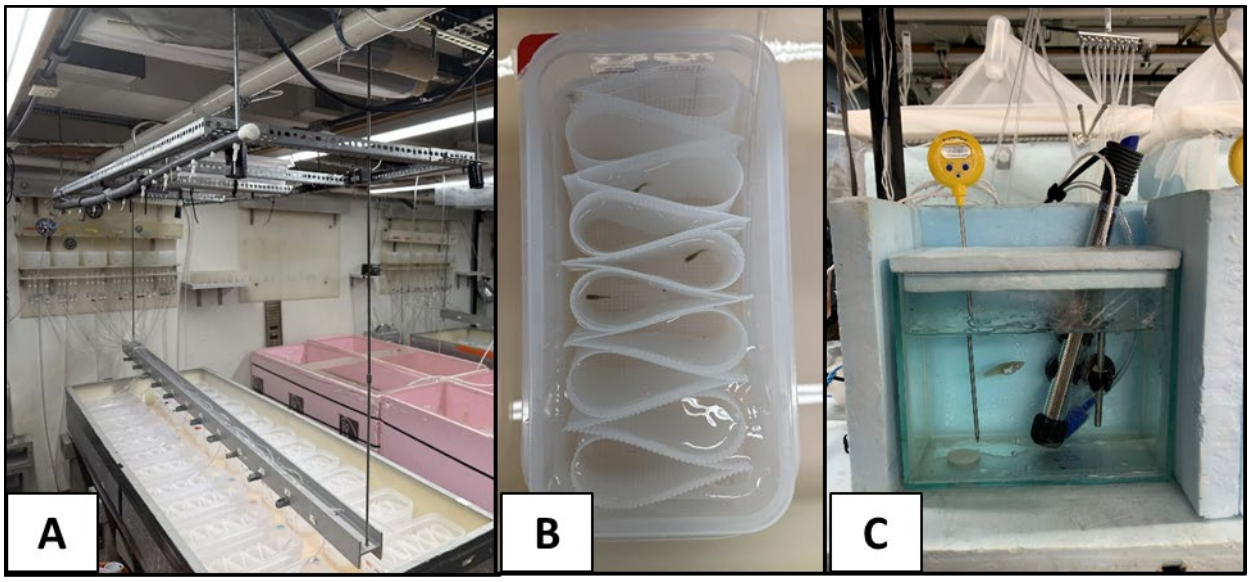


Figure 7.1 A) Continuous flow through dilution system modeled after Benoit et al. 1982. B) Flow through dilution tanks with fish dividers. C) Close up of single experimental critical thermal maxima tank with a female Mosquitofish.

7.2.2 *Toxicant Exposure*

Two continuous flow diluters, modeled after Benoit et al. (1982), were set up to run simultaneously with Mosquitofish in one bay and Plains Topminnow in the other (Figure 7.1.A.). The toxicant was pumped into the head toxicant cell from an eighteen L carboy with 6264 g of anhydrous calcium chloride (CAS: 10043-52-4) dissolved in nanopure water. Calcium chloride was selected as the toxicant of interest for salinity as calcium and chloride were found to be the main cation and anions leading the high salinity concentrations in the lower South Platte River (O'Brien 2020). The toxicant was pumped to the diluter at a rate of 2.5 mL/min using a fine bore peristaltic pump.

Each of the six salinity concentrations (2400 mg/L, 1200 mg/L, 600 mg/L, 300 mg/L, 150 mg/L and control) had four replicate tanks for both species. Western Mosquitofish and Plains Topminnow were housed in allopatry with ten individuals per tank. These individuals were further separated from each other within each tank using plastic mesh screen dividers (Figure 7.1.B.) The exposure period lasted for 30 days, with regular monitoring to ascertain both acute and chronic endpoints (Vosyliene 2007; Desforges et al. 2023). The daily tasks for maintaining the experimental setup focused on monitoring fish and tank conditions, ensuring accurate water chemistry, and feeding fish. Water quality measurements were taken daily using a YSI Pro 1030 to record pH, temperature, and total dissolved solids (TDS) in a rotating block of six tanks. If anomalies in concentrations were detected, the diluter's performance and flow rates were checked and adjusted as necessary. Chloride levels were tested from water samples taken from two tanks in each species bay. Carboys containing calcium chloride ($CaCl_2$) solution were regularly monitored to ensure they held sufficient fluid. Every three days, dissolved oxygen (DO) levels were measured using a YSI ProODO, and tanks were scrubbed and siphoned to maintain cleanliness. Carboys were swapped out as needed, with $CaCl_2$ dissolved in nanopure water for the toxicant solutions. Each day, except days preceding critical thermal maxima experiments, fish were fed once with thawed bloodworms at 10% of the total weight of all individuals in each experimental unit. Food was distributed equally within each divided chamber using a pipette. Mortalities were noted on datasheets, detailing tank number, species, concentrations, and mortality counts.

7.2.3 *Critical Thermal Maxima*

To test for the sublethal effects of temperature fluctuations in saline streams critical thermal maxima was assessed on days four, ten, and thirty. Day four was selected as an acute endpoint while day thirty served as the effect of a chronic endpoint (Suter et al. 1986; Jarvinen et al. 1987). A test was performed on day 10 to test for sublethal effects that may arise before the full thirty-day exposure was completed. A single individual was removed from each experimental tank for critical thermal maxima experiments (i.e., four individuals per concentration and species). To determine the critical thermal maxima (CTM) for each fish species, fish were placed in a controlled tank where the water temperature was gradually increased at a rate of 0.5°C per minute (Figure 7.1C). This slow, steady increase in temperature continued until the fish reached their CTM, which was defined as the point at which they lost equilibrium (i.e., could no longer maintain an upright swimming posture). At this moment, the endpoint temperature was recorded

as the fish's CTM. Immediately after this, the fish were transferred to a recovery tank with cooler water to ensure their survival and recovery from thermal stress. Fish were then euthanized with an overdose of MS-222 and their weight and lengths were recorded. Fish were not returned to the salinity exposure as we did not want to confound the results of the exposure after a stressful CTM event.

7.2.4 *Histopathology*

Plains Topminnow and Mosquitofish sampled for histopathology were first euthanized in buffered MS-222 solution and fixed whole in 10% neutral buffered formalin for >24 hours. They were then bisected on the mid-sagittal plane and processed routinely to histology slides for review by an anatomic veterinary pathologist.

7.2.5 *Data Analysis*

Kaplan-Meier adjusted survival estimates were used to assess the lethality of varying salinity levels on both the Western Mosquitofish and Plains Topminnow survival over the 30-day exposure period. A Kaplan-Meier (Kaplan and Meier 1958) adjustment was applied to censored data, specifically individuals used in the Critical Thermal Maxima (CTM) experiments after 4d and 10d of exposure. The survival distribution was estimated directly from the continuous death times.

An Analysis of Variance (ANOVA) was used to assess the differences in Critical Thermal Maxima (CTM) between treatment levels of salinity and across species, as well as to test for interactions between exposure duration and salinity concentration. In this study, the CTM was measured for each fish after exposure to different salinity concentrations over a 30-day period. Fish were grouped by salinity treatment (six levels of $CaCl_2$) and species (Western Mosquitofish and Plains Topminnow). A two-way ANOVA was performed with species and salinity concentration. This allowed for testing the main effects of both species and salinity on CTM, as well as any interaction between the two factors. In addition, the ANOVA was expanded to include exposure duration (day four, ten, and thirty), testing for an interaction between salinity concentration and exposure time. Tukey's Honest Significant Difference (HSD) tests were used to perform pairwise comparisons between the group means to determine exactly which groups differ from one another when a significant result was found. Tukey's test compares all possible pairs of groups while controlling for Type I error (the probability of falsely identifying a difference). All analyses were performed in R (R Core Team 2024).

7.3 Results

7.3.1 *Salinity Survival*

The results from the Kaplan-Meier survival curve are presented in Figure 7.2. These results show that both Mosquitofish and Plains Topminnow had 100% survivability across all concentrations. Since we saw no significant effect on mortality at any specific concentration, all concentrations are lumped. The survival curves of Mosquitofish and Plains Topminnow are overlain on top of each other.

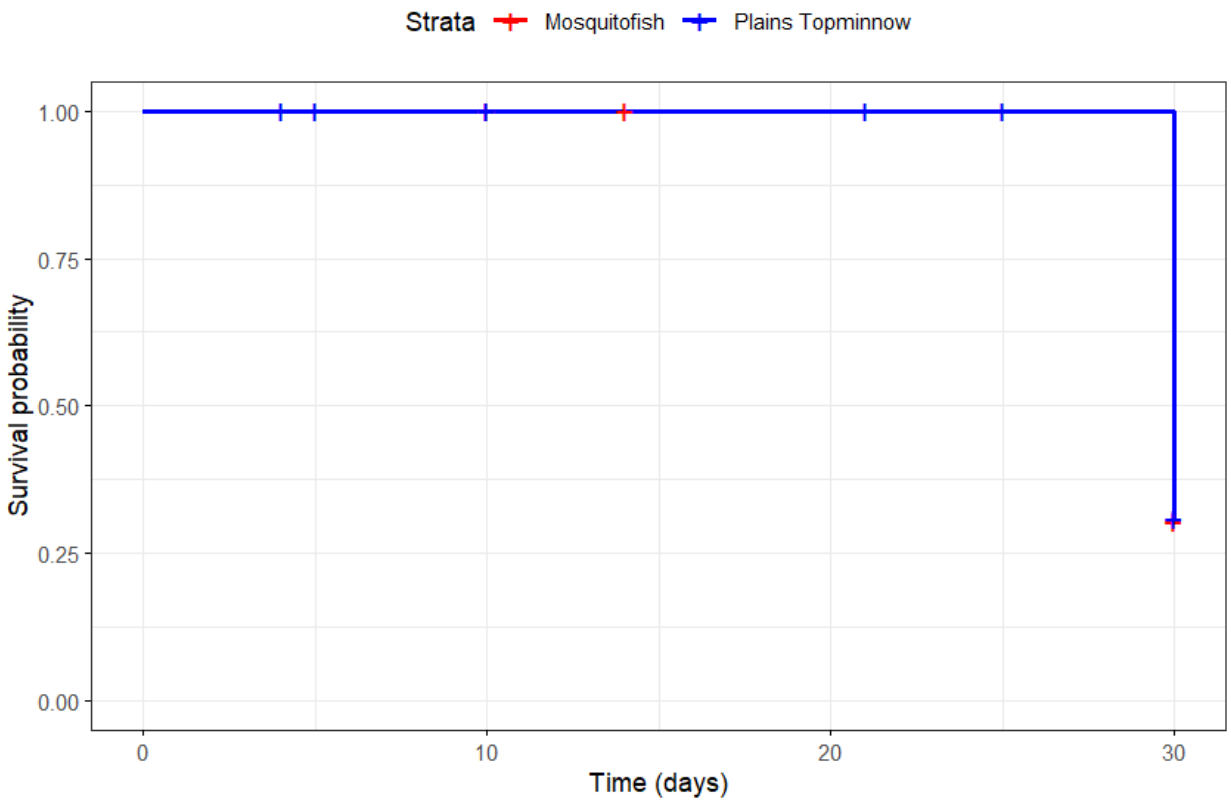


Figure 7.2 Kaplan Meier survival curve for all concentrations for Plains Topminnow (blue) and Western Mosquitofish (red). Survival curves are overlain on top of each other indicating 100% survival at all concentrations across both species for the duration of the study (30 days).

7.3.2 *Critical Thermal Maxima (CTM)*

The critical thermal maxima of Western Mosquitofish (CTM = 35 ºC) was found to be significantly higher (Figure 7.3, $p<0.0001$) than that of the Plains Topminnow (CTM = 32.5 ºC). Concentration was not found to have a significant effect on CTM when accounted for in the two-way ANOVA examining concentration and species. Mosquitofish exhibited reduced thermal tolerance at the highest treatment level. A significant decline in the CTM for Mosquitofish was observed between the highest (2,400 mg/L) and second highest (1,200 mg/L) concentration ($p=$ 0.0044).

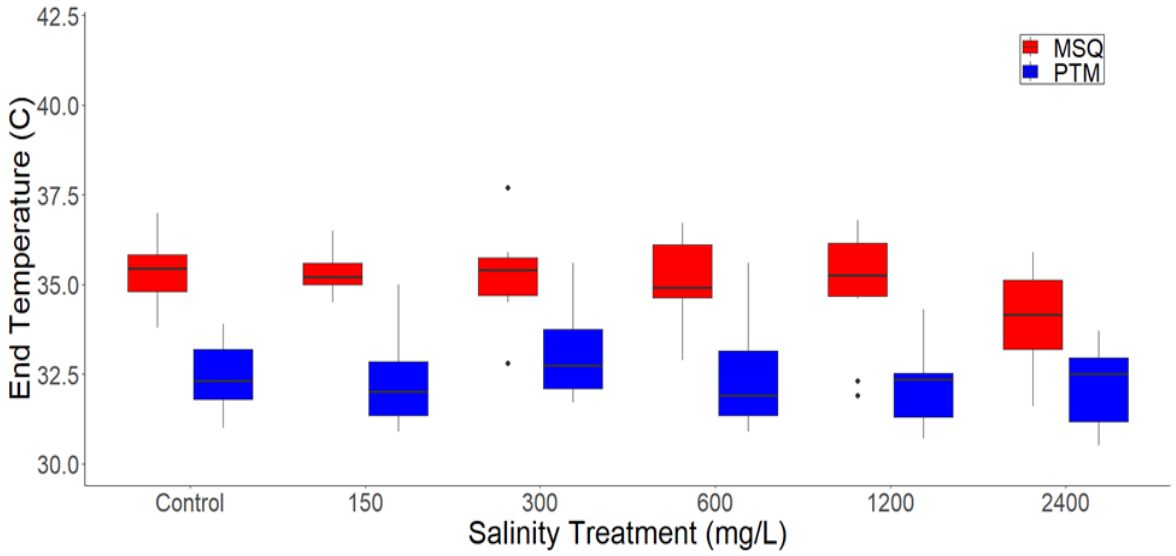


Figure 7.3 Critical thermal maximum results for Plains Topminnow (PTM) and Western Mosquitofish (MSQ) at each of the six experimental concentrations.

Introducing the duration effect in the analysis we observed that the Critical Thermal Maxima of Western Mosquitofish (CTM = 35 ºC) was still found to be significantly higher (p= $2x10^{-16}$) than that of the Plains Topminnow (CTM = 32.5 ºC). With the accounting of duration, we found that concentration (p = 0.023) and duration ($1.71x10^{-17}$) both had significant effects on CTM.

Regardless of duration of exposure (4d, 10d, 30d; Figure 7.4) Western Mosquitofish thermal tolerance (CTM = 35 ºC) was significantly higher (p<0.001) than that of Plains Topminnow (CTM = 32.5 ºC). Both concentration (p= 0.023) and duration (p<0.001) had significant reduction on CTM values for both species.

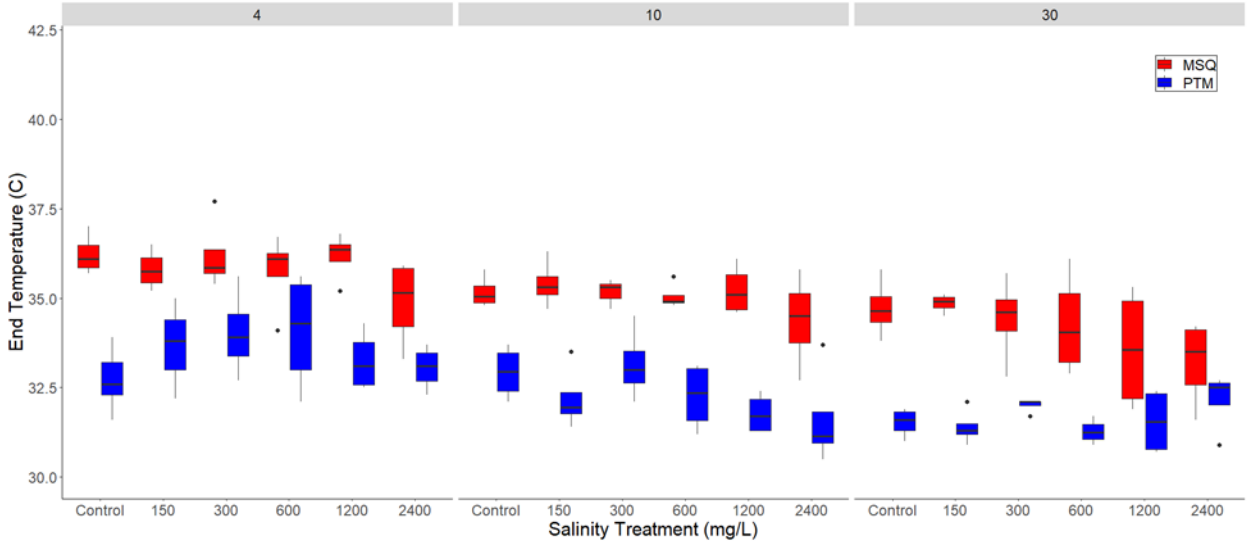


Figure 7.4 Critical thermal maximum for Plains Topminnow (PTM) and Western Mosquitofish (MSQ) on days 4, 10, and 30 at each of the six experimental concentrations.

7.3.3 *Histopathology*

Fifteen control PTM were randomly selected from control tanks, euthanized and preserved for evaluation. Five PTMs randomly selected from treatment tanks (150, 600, and 2,400 mg/L $CaCl_2$) were euthanized, and preserved for evaluation. All together there were fourteen females and sixteen males evaluated. No dose response lesion was identified in any tissue.

Similarly, five control MSQ and five each from the 150, 600, and 2,400 mg/L treatment groups were evaluated histologically. There were eight females, six males, and six unknown sex. There was no relationship to treatment status in any tissue.

7.4 Discussion

Key Findings

Western Mosquitofish's critical thermal maxima is greater than the Plains Topminnow and this largely held true across levels of salinity. These findings suggest that Plains Topminnow is more sensitive to the warming of surface waters than the non-native competitor. Water temperatures are forecast to rise in the face of reduced flows from upstream (diversions and impoundments), lowered water tables from agricultural irrigation, and climate change.

Tolerance to Calcium Chloride was observed in both species. This high tolerance is especially notable for the Plains Topminnow, which is generally considered to be less tolerant to salinity than the invasive Western Mosquitofish. However, these species were mature and large bodied (Figure 7.5). Larger body sized organisms are generally more tolerant to salinity. For this reason the value of these data in the creation of water quality standards is limited. The value of these data in risk assessment and management decisions should mind that fish were mature. This experiment should be repeated with early life stages (post-hatch to 30 d post-swim-up). Fish may have acclimated to the higher salinity levels over the 30-day exposure period. More research is needed to understand the mechanisms underlying this high salinity tolerance in both species.

Figure 7.5 (Left) Experimental tanks with no divider. (Right) Plains Topminnow showing signs of nipped caudal fin from conspecifics. Note large body size.

7.5 References

Alcaraz, C., A. Bisazza, and E. García-Berthou. 2008. Salinity mediates the competitive interactions between invasive Mosquitofish and an endangered fish. Oecologia 155(1): 205-213.

Benoit, D. A., V. R. Mattson, and D. C. Olsen. 1982. A continuous flow mini-diluter system for toxicity testing. Water Research 16: 457–464.

Cuthbert, R. N., Z. Pattison, N. G. Taylor, L. Verbrugge, C. Diagne, D. A. Ahmed, B. Leroy, E. Angulo, E. Briski, C. Capinha, J. A. Catford, T. Dalu, F. Essl, R. E. Gozlan, P. Haubrock, M. Kourantidou, A. M. Kramer, D. Renault, R. J. Wasserman, and F. Courchamp. 2021. Global economic costs of aquatic invasive alien species. Science of the Total Environment 775: 145238.

Desforges, J. E., K. Birnie-Gauvin, F. Jutfelt, K. M. Gilmour, E. J. Eliason, T. L. Dressler, D. J. McKenzie, A. E. Bates, M. J. Lawrence, N. Fangue, and S. J. Cooke. 2023a. The ecological relevance of critical thermal maxima methodology for fishes. Journal of Fish Biology 102(5): 1000-1016.

Desforges, J. E., K. Birnie-Gauvin, F. Jutfelt, K. M. Gilmour, E. J. Eliason, T. L. Dressler, D. J. McKenzie, A. E. Bates, M. J. Lawrence, N. Fangue, and S. J. Cooke. 2023b. The ecological relevance of critical thermal maxima methodology for fishes. Journal of Fish Biology 102(5): 1000-1016.

Dodds, W. K., K. Gido, M. R. Whiles, K. M. Fritz, and W. J. Matthews. 2004. Life on the edge: The ecology of Great Plains prairie streams. BioScience 54(3): 205.

Falke, J. A., K. D. Fausch, R. Magelky, A. Aldred, D. S. Durnford, L. K. Riley, and R. Oad,.2011. The role of groundwater pumping and drought in shaping ecological futures for stream fishes in a dryland river basin of the western Great Plains, USA. Ecohydrology 4(5): 682-697.

García-Berthou, E. 2007. The characteristics of invasive fishes: What has been learned so far? *J. Fish Biol.* 71(sd): 33-55.

Gessner, M. O., and A. Tlili. 2016. Fostering integration of freshwater ecology with ecotoxicology. Freshwater Biology. 61(12): 1991-2001.

Hopper, G. W., K. B. Gido, C. A. Pennock, S. C. Hedden, B. D. Frenette, N. Barts, C. K. Hedden, and L. A. Bruckerhoff. 2020. Nowhere to swim: Interspecific responses of prairie stream fishes in isolated pools during severe drought. Aquatic Sciences. 82(2): 42.

Hosseini, P., and R. T. Bailey, R. T. 2022. Investigating the controlling factors on salinity in soil, groundwater, and river water in a semi-arid agricultural watershed using SWAT-Salt. Science of the Total Environment 810: 152293.

Kaplan, E. L., and P. Meier. 1958. Nonparametric estimation from incomplete observations. Journal of the American Statistical Association. 53(282): 457-481.

Kelley, A. L. 2014. The role thermal physiology plays in species invasion. Conservation Physiology 2(1): cou045.

Laha, M., and H. T. Mattingly. 2006. Identifying environmental conditions to promote species coexistence: An example with the native Barrens Topminnow and invasive Western Mosquitofish. Biological Invasions 8(4): 719-725.

Lee, C. E. 2003. Evolution of physiological tolerance and performance during freshwater invasions. Integrative and Comparative Biology 43(3): 439-449.

Lennox, R., K. Choi, P. M. Harrison, J. E. Paterson, T. B. Peat, T. D. Ward, and S. J. Cooke. 2015. Improving science-based invasive species management with physiological knowledge, concepts, and tools. Biological Invasions 17(8): 2213-2227.

Strange, M. (present address: Stratus), K. D. Fausch, and A. P Covich. 1999. Sustaining ecosystem services in human-dominated watersheds: Biohydrology and ecosystem processes in the South Platte River basin. Environmental Management 24(1): 39-54.

Pyke, G. H. 2008. Plague minnow or mosquito fish? A review of the biology and impacts of introduced Gambusia species. Annual Review of Ecology, Evolution, and Systematics 39: 171-191.

Reeve, A. J. n.d. Phenotypic plasticity in thermal tolerance – life history strategy of an invasive freshwater fish.

South Platte River salinity report – January 2020 – rev 1.pdf. n.d. Retrieved October 6, 2022, from https://www.dropbox.com/s/lh894u5pzl130i6/South%20Platte%20River%20Salinity%20Report-January%202020-rev1.pdf?dl=0&unfurl=1

Sprague, L. A. 2005. Drought effects on water quality in the South Platte River basin, Colorado. Journal of the American Water Resource Association 41(1): 11-24.

Zhou, L., X. Ouyang, Y. Zhao, G. Gomes-Silva, S. I. Segura-Muñoz, J. Jourdan, R. Riesch, and M. Plath. 2022. Invasive fish retain plasticity of naturally selected, but diverge in sexually selected traits. Science of the Total Environment. 811: 152386.