Water Pollution Studies

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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 long after disturbance. If not directly informed by experiments using native Colorado species, changes to site specific and state wide water quality standards risk compromising fisheries

health. Decision makers and regulators need to be informed of risks to Colorado specific species and aquatic communities. These organisms are often underrepresented in scientific literature. Efforts to restore Colorado's endangered fish species often require precise use of piscicides which are difficult to assess in the field. In the last decade, the unique analytical capabilities of the CPW Aquatic Toxicology Laboratory have been employed to provide this information on short turnaround using a mobile laboratory. Collaborators at state agencies and universities frequently approach ecotoxicological topics that concern CPW's fish and wildlife. However, rarely is this research usable in the water quality standard building framework of the USEPA and CDPHE. By collaborating with these researchers and agencies CPW can ensure research considers the unique needs of our agency and water quality regulatory agencies. By sharing equipment, labor, and infrastructure, these collaborative projects produce better data that is more useful to CPW's mission. Technical support conducted by the CPW Aquatic Toxicology Laboratory includes, but is not limited to:

1. Provide technical assistance and expertise, consultation, evaluation and training in aquatic toxicology and aquatic chemistry to Colorado Parks and Wildlife and other state and federal personnel as requested.
2. Assist in the investigation of fish kills.
3. Conduct short- or long-term experiments to produce toxicity data or develop site-specific field studies to address local management decisions or local site-specific variances, when such data in the literature are lacking or inadequate.
4. Collect and analyze water and/or fish tissues to assess water quality problems as requested.
5. Analyze rotenone (and other piscicides) in water samples as part of Colorado Parks and Wildlife reclamation projects.
6. Publish and review results of experiments and water quality investigations in peer-reviewed journals for consideration in policy making by other agencies.

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Assessment of *Renibacterium salmoninarum* Prevalence in Pathogen Loads in Wild Brook Trout Populations and the Implications for the Stocking of YY Males

Conservation of the Uncompahgre Cutthroat Trout (*Oncorhynchus clarkii pleuriticus*) in Bobtail and Steelman creeks is threatened by the invasion of nonnative Brook Trout (*Salvelinus fontinalis*). While intensive mechanical removals have been ongoing, an alternative strategy using genetically YY male Brook Trout was considered to induce population collapse through sex ratio manipulation. YY Brook Trout from a hatchery were created to stock into these populations. A key concern with this approach was the potential for introducing new pathogens, specifically *Renibacterium salmoninarum*, which causes bacterial kidney disease. To address this, we conducted a study to determine the prevalence of *R. salmoninarum* in the Brook Trout and current Cutthroat Trout populations located in Bobtail and Steelman Creeks before introducing YY males that were coming from a potential *R. salmoninarum*-positive hatchery source. We collected kidney, liver, and spleen tissue from Brook Trout and Cutthroat Trout and analyzed the samples using both quantitative polymerase chain reaction (qPCR) to detect bacterial DNA and enzyme-linked immunosorbent assay (ELISA) to detect bacterial antigens. While qPCR showed a low prevalence of *R. salmoninarum* in both creeks (8.8% in Steelman and 11.7% in Bobtail), the ELISA consistently detected a much higher prevalence of antigens, suggesting widespread exposure. In Steelman Creek, antigen prevalence was 93% in Brook Trout and 100% in Cutthroat Trout, and in Bobtail Creek, it was 100% in Brook Trout. Our findings confirm that *R. salmoninarum* is already present in these wild populations. This result alleviates concerns that the introduction of YY male Brook Trout would bring a novel pathogen into the watershed and supports the continued development using YY fish for Brook Trout suppression.

Chemical Avoidance

Chemical avoidance studies examining road salts are ongoing. Loss of fish in streams adjacent to highways has been reported across Colorado. When only mortality is measured, fish are found to be extremely tolerant to magnesium chloride ($MgCl_2$) and sodium chloride (NaCl). But fish can sense and avoid low levels of $MgCl_2$ and NaCl. Colorado mountain streams fall down steep gradients that limit fish passage upstream. Barriers such as culverts, diversion structures, whitewater parks, reservoirs and spillways all prevent upward migration. Chemical avoidance in Colorado lotic systems can result in downstream “drift” which will possibly result in loss of a species at sublethal levels. Colorado Parks and Wildlife has been devising annular chemical preference chambers and perfecting chemical avoidance trials for part of this fiscal year. See Chapter 2 of this report for examples of fish avoidance of road salts. In the near future this will be expanded to other salts, zinc, pH, chlorine, bicarbonate, ammonia, sulfate, and other toxicants.

Differences between Constant Temperatures and Diel Temperature Fluctuations for Fish Temperature Tolerance

Colorado's water quality regulations protect fish populations by setting temperature standards designed to support their physiological processes, including growth, metabolism, and survival. Currently, chronic temperature standards rely on scientific studies that determine optimum and tolerable temperatures by holding fish at constant water temperatures over the course of an experiment. However, the community responsible for temperature regulations has raised concerns that these standards may be unreliable because wild fish experience diel temperature fluctuations. To address this concern, we conducted a literature review to compare studies using constant versus fluctuating water temperature to determine their effect on optimum and tolerable temperatures for fish. Our search resulted in an analysis of 21 relevant studies. While a definitive strong trend remains unobserved, the majority of studies focusing on optimum growth suggest that fluctuating temperatures may lower fish growth rates compared to constant temperatures. Conversely, there was no consistent pattern found among studies investigating the effects of temperature fluctuations on fish metabolism. The analysis of this literature review is ongoing and will culminate in a white paper. This report will be used to determine if Colorado's current chronic temperature standards are under-protective or over-protective due to their heavy reliance on constant water temperature studies for establishing optimum temperatures.

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 with adults, rather than traditional lethal 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. Studies are currently underway to conduct tests on Bluehead Suckers and Flannelmouth Suckers from the Gunnison River and hatchery fish.

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 *P. 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.

Glomerulonephritis in Plains Topminnow

Details regarding the diseases of freshwater native small-bodied fish species are sparse and little is known about their potential effects on survival and population persistence. Spurious mortality was observed in field collected Plains Topminnow *Fundulus sciadicus* (PTM) intended for toxicology and temperature experiments. Experimental use for this population was canceled due to concerns of organisms' health. A high incidence of kidney disease was incidentally noted by histopathology in PTMs submitted to the Colorado State University Veterinary Diagnostic Laboratories. PTM were scored for the presence/absence of glomerulonephritis, renal tubular mucus, tubular epithelial attenuation, necrosis, and regeneration, tubular mineral, neonephrogenesis, and the presence of renal myxozoans. One hundred nineteen of 146 PTM kidneys (82%) had global proliferative glomerulonephritis with synechiae (81%), fibrosis (76%), cystic dilation of Bowman's capsule (18%), and rare fibrinoid crescents (5%). Tubular mucus (88%), renal tubular epithelial attenuation (74%), tubular epithelial cell necrosis (60%), and tubular mineral (29%) were common, as were tubular regeneration (86%) and neonephrogenesis (35%). We suspect that the high prevalence of tubular regeneration and neonephrogenesis reflects a reparative response, and that the capacity for regeneration allows the PTMs to survive. These fish may be able to compensate as long as regenerative capacity equals or exceeds the pace of nephron impairment and destruction. The energetic cost required to compensate for the consequences of chronic kidney disease (e.g., proteinuria) and to sustain regeneration and neonephrogenesis is unknown. This chronic stress may be additive or synergistic with toxicant stress. Transmission electron microscopy on five kidneys demonstrated podocyte injury and lack of electron dense deposits in glomerular basement membranes. Myxospores consistent with the genus *Myxobolus* were observed in 63% of fish and were more common in the kidney interstitium (45%) than glomeruli (23%) where they formed quiescent xenomas and rarely colocalized with other glomerular pathology; thus, they are not proposed to be the cause of glomerulonephritis. The small subunit ribosomal DNA sequence was ≤ 95 % similar to

Myxobolus spp. from North American freshwater fishes. The sequence divergence from congeners, myxospore morphometrics, renal tropism, and fish host support a diagnosis of a novel species. Renal disease is poorly described in teleost fishes and greater efforts are needed to determine the causes and impacts on fish fitness and population survival, especially in threatened species. Interactions between parasite load and toxicants warrants increased study, especially parasites that induce loss of kidney function.

Lethal and Sublethal Effects of Magnesium Chloride Exposures to Cutthroat Trout

Freshwater salinization, typically driven by road salt runoff, is a growing concern to aquatic ecosystems. While previous research has focused on the lethal effects of salt exposure, the sublethal impacts on aquatic life remain poorly understood. This study investigates the lethal and sublethal effects of magnesium chloride ($MgCl_2$) with various life stages of Cutthroat Trout *Oncorhynchus clarkii stomias*, a Tier 1 native species to high-elevation streams. We conducted a series of acute toxicity tests on three age classes, 30-day post-swim up (d psu), 6-month-old juveniles, and one-year-old fish, where we measured mortality, water-borne cortisol (a stress hormone), and thermal tolerance with critical thermal maximum (CTMax). Our results showed that $MgCl_2$ was not acutely lethal to any age class at the concentrations we tested, with only one mortality occurring at the highest concentration (2,500 mg L^{-1}). One-year-old fish were highly resilient, showing no significant changes in cortisol levels or CTMax. Interestingly, cortisol levels were significantly higher in high-density tanks, suggesting that crowding was a greater stressor than $MgCl_2$ exposure. The 30 d psu fish were the most sensitive stage and showed a significant reduction in thermal tolerance at the highest concentration (860 mg L^{-1}). This suggests a sublethal effect in the absence of mortality. These findings highlight the importance of assessing sublethal endpoints across different life stages, and they indicate that exposure to $MgCl_2$ based road salt may compromise the physiological resilience of Cutthroat Trout to other environmental stressors like increasing temperatures.

Sinoatrial *Contracaecum* spp in Plains Topminnow

Spurious mortality was observed in field collected Plains Topminnow *Fundulus sciadicus* (PTM) intended for toxicology and temperature experiments. Large nematode worms were identified histologically in Plains Topminnow (PTM) gathered from Willow Creek, a South Platte tributary on the Pawnee National Grasslands, Weld County, Colorado. Prospective dissection and histopathology identified sinoatrial worms in 22 of 151 PTMs (14.5%). Nematodes recovered by dissection were evaluated by a veterinary parasitologist and key genes were sequenced to allow for taxonomic identification. Parasitologic evaluation identified a mucron and boring tooth indicative of a larval Anasakid and sequencing analysis identified the worm as belonging to the *Contracaecum* genus.

The worms caused marked aneurysmal dilation of the sinus venosus and less frequently extended cranially into and seemingly occluded the cardinal vein. Intravascular nematodes in many species (both fish and mammals) are detrimental to the host and many cause heart failure. If *Contracaecum* is detrimental to PTM fitness this may be a factor to consider alongside ecological threats in overall conservation goals. Reduced aerobic health is an obvious result of large bodied parasites in the heart of a small-bodied fish. This could have direct implications on heat tolerance, low dissolved oxygen tolerance, dispersal, and predator avoidance. Reduced

immune function is a likely effect after chronic exposure to toxicants present in PTM historical range (*e.g.* insecticides, herbicides, fungicides, fertilizers). Parasite load is a possible indicator of reduced immune function. The synergistic and additive effects of toxicants and parasites deserve further study. Findings were published in the journal *Aquaculture, Fish and Fisheries.*

On-site Diagnostic Tools for Estimating Fish Exposure to Aqueous Metals

The Colorado Mineral Belt hosts a high density of river systems with high concentrations of aqueous metals. Some basins have seen accelerated weathering and release of metals due to anthropogenic activity (e.g. mining, tunnel and road waste rock/fill). Even those streams void of anthropogenic disturbance experience metal rich parent material slowly releasing aqueous metals. Anthropogenic climate change is likely to increase warm weather season duration and temperatures in alpine and tundra habitats. These high mountain peaks historically remained frozen and even ice covered for a majority of the year. The increased temperature and increased percolation of water will increase metal concentrations in upper and lower reaches regardless of legacy mines. Frequency of fish kills in the Roaring Fork Valley has become problematic. Similar events are likely to become more frequent in the Colorado mineral belt.
Human blood chemistry instruments have become miniaturized in recent years to allow instant bed size results. Microscopy, cytology, vital dye florescence, and element specific staining techniques are possible in the recently acquired CPW mobile toxicology laboratory. Rapid and reliable streamside assessment techniques would be useful not only for fish kill response but also to assess chronic or recent acute exposures to aqueous metals. These can be paired with confirmatory diagnostics (histopathology) and metal concentration assessed by digestion and ICP-MS

Cytology based histochemical tests for metals were compared to histopathology and tissue quantitation of metals. Fish were collected from Iowa Gulch and McNasser Creek near Leadville, CO and Lincoln Creek near Aspen, CO. The following were collected from each organisms: gill cytology, gill for histopathology, gill for quantitation; liver cytology, liver for histopathology, liver for quantitation; kidney cytology, kidney for histopathology, and kidney for quantitation. Blood smears and ocular fluid were also collected. Packed cell volume was determined in the field. Metal analyses of digested tissue by ICP-MS are pending.

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 well-being. 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.

Vital Dye Image Analysis for Lateral Line Morphologic and Toxicologic Application

The lateral line is a key sensory organ comprised of many individual neuromasts that are distributed on and within fish skin. This system helps to sense fluid flow and allows for fish behaviors such as shoaling, Karman gaiting, foraging, and evasion of predation. Neuromasts offer analogous structure for human inner ear cells, so they have been well characterized and recently used as a model for human ototoxicity. CPW and Colorado State University's Veterinary Diagnostic Laboratory are applying these human medicine methodologies to fish conservation. We specifically wished to extrapolate vital dye methodology for lateral line morphology and toxicity. Fluorescent vital dyes such as 4-Di-2-Asp are selectively absorbed by living neuromasts, which allows the distribution of neuromasts to be mapped in normal or abnormal fish. Injured, degenerate, or dead neuromasts do not take up the dye, a response variable that can be used in toxicology studies. We tested vital dye concentrations, adapted a dissecting microscope with fluorescence microscopy, and determined best methods for image capture and image analysis. Further refinement will make this microscopy non-lethal. We have successfully applied this technique to further study effects of diesel fuel spills (See Chapter 4) in the laboratory. Use of this technique for Cobalt toxicity trials is ongoing.

Vulnerability Assessment of 6PPD-q Exposure in Colorado's Aquatic Ecosystems for Tier 1 Species Conservation

The breakdown product of the widely used tire additive 6PPD, 6PPD-quinone (6PPD-q), is an emerging contaminant of concern in aquatic ecosystems. It is known to be acutely toxic to certain fish species, and its presence in runoff from roadways poses a direct threat to freshwater habitats. In Colorado, this presents a particular risk to Tier 1 aquatic species such as the Cutthroat Trout and Boreal Toad. Our research aims to identify and sample locations in Colorado with the highest risk of 6PPD-q runoff. We use a predictive modeling approach with ArcGIS Pro and Rstudio to pinpoint these high-risk areas, or "hotspots." Our model integrates a range of data including road density, traffic volume, precipitation patterns, and watershed characteristics, alongside the known locations of these Tier 1 species. Once complete, we will assign a biological vulnerability score to each watershed (HUC12) in Colorado based on these factors, creating a composite risk map that visualizes areas where 6PPD-q exposure is most likely to harm sensitive species. Using this map, we can then strategically collect water samples after storm runoff events and determine the concentrations of 6PPD-q in our waterways.

Water Quality Assessment of Gunnison River Tributaries

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 suckers 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 have been evaluating 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.

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). Assistance in benthic sampling and terrestrial insect sampling was conducted in Arkansas River near Leadville, CO.

Feasibility Studies for Laboratory Relocation

Laboratory staff Megan McConville, Tawni Firestone, Riley Halford, Mathew Bolerjack and Pete Cadmus have engaged in cataloging and measuring of laboratory space, infrastructure and processes for including in a relocation feasibility study being conducted by a vendor selected by CPW's engineering staff.

Fish Kill Response

CPW Toxicology Laboratory gave in-person sampling assistance, autopsy service, chemical analysis or chemical sampling, or over-the-phone consulting in response to four major fish kill or spill events and numerous smaller fish kill and spill events.

Hatchery Water Quality Testing

A statewide hatchery water quality testing at all production water sources was implemented 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 and low flow fall 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), cadmium (Cd), copper, (Cu), 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 27 water quality data hatchery reports have been analyzed in FY25 and reported to the water quality section for quality assurance and quality control.

Information Sheet: Understanding and Utilizing Onsite HPLC Analysis at Rotenone-based Reclamation Projects

An information sheet was created and sent to biologists planning reclamation projects for 2025. This document outlines how an on-site HPLC can be used to improve the efficiency and success of rotenone-based reclamation projects. The document highlights that rotenone is a natural piscicide used in fisheries management that works by disrupting cellular energy production in fish. High performance liquid chromatography (HPLC) is a field-deployable analytical tool used to accurately measure the precise concentration of rotenone, complementing traditional field methods such as using sentinel fish. Biologists can use this document to understand how to use the rotenone analysis data on their projects. Specifically, it outlines the following:

- Verifying application concentrations: On-site HPLC provides immediate, real-time data to confirm that all drippers and spray crews are delivering the intended concentrations of rotenone. This is particularly valuable on the first day of treatment.
- Troubleshooting and targeting organic-rich areas: The analysis can quickly identify areas where rotenone concentrations are lower than expected due to binding with organic matter. This allows biologists to immediately adjust the dose or add more rotenone to these “hot spots” to ensure adequate fish mortality.
- Assessing detoxification effectiveness: By sampling both above and below the detoxification point, biologists can continuously monitor the rotenone plume and confirm that the potassium permanganate is effectively neutralizing the chemical. This allows for immediate adjustments to the detox drip rate as needed.
- Confirming plume travel time: Strategic sampling can be used to confirm travel time calculations for the rotenone plume, ensuring that the detox station is correctly positioned and activated to prevent the spread of the chemical to non-target areas.

This document also provides guidance on sampling, noting that the number of samples collected depends on the project’s scope, with a typical range of 50-100 samples over a two-day treatment period.

In summary, the information sheet emphasizes that implementing on-site HPLC analysis provides biologists with real-time, precise information. This allows for informed decisions that lead to a more effective, efficient, and environmentally responsible application of rotenone for successful fisheries management and ecosystem restoration.

Milt Extender Production
Milt extender was produced for federal and state natural resource management agencies across the country March of 2025.

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: Trinchera Ranch Reclamation Project (Fort Garland, CO: July 2024), Lost Dog Creek Reclamation Project (Steamboat, CO: August 2024), and Slater Lake Reclamation Project (Idaho Springs, CO: September 2024).

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. Asterisks represent corresponding author.

- **Firestone, T. B. R.***, R. Walters, B. Felt, T. Swarr, K. Morben, M. Baker, M. May, and M. McConville. 2025. Challenges of eradication efforts for Zebra Mussels in Highline Lake, Colorado using EarthTec QZ® – In Press *Management of Biological Invasions*
- **Firestone, T. B. R.***, E. R. Fetherman, and D. L. Winkelman. 2025. Non-lethal detection of *Renibacterium salmoninarum* in Cutthroat Trout *Oncorhynchus clarkii* comparing mucus, blood, and ovarian fluid samples to kidney tissues. *Journal of Aquatic Animal Health.* doi: 10.1093/jahafs/vsaf013 **Editors Choice*
- **Firestone, T. B. R.***, E. R. Fetherman, K. Huyvaert, J. Drennan, R. E. McDevitt, B. Yeatts, and D. L. Winkelman. 2025. Leveraging detection uncertainty to estimate *Renibacterium salmoninarum* infection status among multiple tissues and assays. *PLOS One 20(4): e0323010.* doi: 10.1371/journal.pone.023010
- Schaffer, P. A.*, A. K. McGrew, J. Henley, C. M. Adams, D. L. Winkelman, R. M. Fitzpatrick, **P. Cadmus**. 2025. Sinoatrial Contracaeciasis in Johnny Darters (*Etheostoma Nigrum*) and Plains Topminnow (*Fundulus Sciadicus*) from the South Platte Drainage, Colorado. Aquaculture, Fish and Fisheries 5:e70100 https://doi.org/10.1002/aff2.70100
- Dils R., **T. B. R. Firestone***, P. A. Schaffer, E. R. Fetherman, and D. L. Winkelman. 2024. *Renibacterium salmoninarum* in Chinook Salmon (*Oncorhynchus tshawytscha*) following intraperitoneal injection: Description of histological progression and bacterial load dynamics. *Diseases of Aquatic Organisms* doi: 10.3354/dao03852

Professional Reports:

- **Firestone, T. B. R.** and D. Kowalski. 2025. Research results for supporting BKD stocking regulations. Colorado Parks and Wildlife, Aquatic Research Section.
- **Firestone, T. B. R.**, E. R. Fetherman, and D. L. Winkelman. 2025. Detection methods for *Renibacterium salmoninarum*. Aquatic Research Science Briefing. Colorado Parks and Wildlife, Aquatic Research Section.
- **Firestone, T. B. R.**, Z. E. Hooley-Underwood, and **P. Cadmus**. 2025. Bluehead Sucker and Flannelmouth Sucker temperature tolerance. Aquatic Research Science Briefing. Colorado Parks and Wildlife, Aquatic Research Section.
- **Cadmus, P.**, **T. B. R. Firestone**, D. Woller, and R. Dils. 2024. Water Pollution Studies. Annual Report. Colorado Parks and Wildlife, Aquatic Research Section.
- Rust, A., M. McConville, M. May, J. Logan, T. Swarr, J. Ewert, **T. Riepe**, **P. Cadmus** and R. Harris. 2024. Examining changes to an alpine wetland and freshwater ecosystem of Straight Creek, Colorado. Colorado Parks and Wildlife White Paper.

Internal presentations to CPW staff were used to update managers on current research and inform management decisions in Colorado.

- **Firestone, T. B. R**. 2025. Road Salt Experiment Updates. Water Quality Section and Aquatic Biologists. Virtual. March 19, 2025.
- **Firestone, T. B. R.** and P. Cadmus. 2025. Ecotoxicology: A Necessity for Biodiversity Conservation. Colorado Parks and Wildlife, Aquatic Research Section. Virtual. February 25, 2025.

External presentations provided an opportunity to give research updates to managers both within and outside of Colorado and to students at universities.

- Fetherman, E. R., R. Brock, R. Dils, B. W. Avila, and **T. B. R. Firestone**. 2025. Susceptibility of XX, XY, and YY Brook Trout *Salvelinus fontinalis* to infection by *Renibacterium salmoninarum*. Western Division of the American Fisheries Society. Westminster, CO. May 15, 2025.
- Woller, D. U., **T. B. R. Firestone**, and D. L. Winkelman. 2025. Comparing field-based temperature standards to laboratory derived standards for Bluehead and Flannelmouth Suckers. Western Division of the American Fisheries Society. Westminster, CO. May 14, 2025.
- **Cadmus, P.**, M. Moore, and R. Halford. 2025. Roles and responsibilities in the field of water quality – policy and career opportunities. River Science and River Watch Career Development Incentive Program (CDIP). Del Norte, Colorado April 22, 2025.
- **Cadmus, P**., M. Moore, and M. Bolerjack. 2025 A primmer on assessing water quality for future fish biologists. Colorado State University Student Subunit of the American Fisheries Society. Fort Collins, Colorado. February 6, 2025.
- **Firestone, T. B. R**. 2024. Integrating aquatic toxicology and disease research for ecosystem health and species conservation. Western State University Student Subunit of the American Fisheries Society. Gunnison, CO. December 12, 2024.
- Woller, D. U., **T. B. R. Firestone**, and D. L. Winkelman. 2024. Comparing field-based temperature standards to laboratory derived standards for Bluehead and Flannelmouth Suckers. Desert Fish Council Meeting. Grand Junction, Colorado. November 21, 2024.
- **Riepe, T. B.**, R. Walters, M. Baker, T. Swarr, B. Felt, K. Morben, M. McConville, M. May, and A. Rust. 2024. The use of EarthTec QZ® treatment in a Colorado Reservoir to control invasive Zebra Mussels. American Fisheries Society National Meeting. Honolulu, HI. September 19, 2024.
- **Riepe, T. B**. 2024. Addressing complex toxicology challenges in aquatic ecosystems. American Fisheries Society National Meeting. Honolulu, HI. September 17, 2024.
- Lewis, S. T., **P. Cadmus**, J. D. Salerno, and Y. Kanno. 2024. Ecotoxicological evaluation of salinity's effects on invasive Mosquitofish and a Native Topminnow. American Fisheries Society Annual Meeting. Honolulu, Hawaii. September 16, 2024.
- Schaffer, P.A., A. McGrew, and **P. Cadmus**. 2024. Sinoatrial Contracaeciasis in two Johnny darters (*Etheostoma nigrum*) and three Plains topminnow (*Fundulus sciadicus*) from the South Platte Drainage, CO. Rocky Mountain Conference of Parasitologists.

Assessment of *Renibacterium salmoninarum* Prevalence and Pathogen Loads in Wild Brook Trout Populations and the Implications for Stocking of YY Males

Dr. Tawni B. R. Firestone, Riley Dils, Dr. Kevin Rogers, Dr. George Schisler

1.1 Introduction

Bobtail and Steelman Creeks, located in the headwaters of the Williams Fork of the Colorado River near the Continental Divide, are home to two core conservation populations of the Uncompahgre Cutthroat Trout (UPCT). This lineage was formerly known as the green lineage of Colorado River Cutthroat Trout *Oncorhynchus clarkii pleuriticus* (Metcalf et al. 2012; Rogers et al. 2018; Bestgen et al. 2019; Rogers et al. 2025). Conservation of this trout lineage is a high priority for Colorado Parks and Wildlife. Only 69 populations remain across their native range, and just 43 are considered genetically unaltered, a requirement for core population status (*sensu* UDWR 2000; Hirsch et al. 2013, Rogers et al 2020). These two populations have been isolated for over 80 years by a water diversion system completed in 1940. Sluicing operations to clear the diversion structures have allowed nonnative Brook Trout *Salvelinus fontinalis* to invade the upstream Cutthroat Trout populations, with the first invasion documented in the mid-1980s (D. Conklin, GEI, personal communication). Left unchecked, Brook Trout will completely replace Cutthroat Trout where they coexist (Peterson et al. 2004; Separd 2004; Fausch et al. 2009). By the turn of the century, Brook Trout constituted 68% of the trout in Bobtail Creek and 47% in Steelman Creek (unpublished data). This prompted occasional mechanical removal efforts to suppress their populations. These initial efforts proved inadequate to halt the invasion (Peterson et al. 2008), leading to the initiation of more comprehensive annual basin-wide removals in 2011. These sustained removal efforts have helped ensure the persistence of the native trout populations until a larger-scale reclamation is completed.

Original plans for securing these two trout populations involved a multi-stage approach. First, large concrete migration barriers were installed below the existing water diversion structures on both streams. Following this, a reclamation was scheduled at the nearby McQueary Lake drainage using rotenone to eliminate nonnative trout. A subset of Bobtail and Steelman Creek fish were to be translocated into the resulting vacant habitat to serve as a wild brood source if additional fish were required to repopulate the entire watershed. Remaining Cutthroat Trout in Bobtail Creek were to be salvaged and translocated to Steelman Creek so the former could be treated with rotenone to eliminate the resident Brook Trout. Fish from Steelman Creek were then to be repatriated to Bobtail Creek following treatment so the process could be repeated in the former. This phased, basin-wide approach was projected to take over a decade to implement and require substantial effort and cost.

As the planning process for the Bobtail and Steelman Creeks unfolded, a promising alternative technology for eradicating Brook Trout emerged. Idaho has pioneered the use of genetically YY male Brook Trout, which, when introduced into a population, skew the sex ratio toward 100% males, leading to a population collapse (Schill et al. 2016). These YY males, genetically distinct

from the typically XY male, pass on a Y chromosome to all their offspring, ensuring that any surviving young are also male. By continuously introducing enough YY males into a system, the sex ration can be manipulated to the point where the population cannot sustain itself, eventually leading to its eradication (Teem and Gutierrez 2010; Schill et al. 2017). Bobtail and Steelman Creeks are ideal candidates for this approach because they are small streams (<3.5 km) and are already subject to previous intensive mechanical removal efforts (Schill et al. 2017; Kennedy et al. 2018; Davenport et al. 2024).

Introducing fish into a conservation population can raise concerns about pathogen transmission. Here, a key concern is the potential for introducing *Renibacterium salmoninarum*, the bacterium responsible for bacterial kidney disease in salmonids. This pathogen is particularly successful in infecting its hosts because it can be transmitted both vertically and horizontally (Balfry et al. 1996; Evelyn et al. 1986). The YY male Brook Trout we planned to stock in Bobtail and Steelman Creeks were sourced from a facility with a past history of *R. salmoninarum* infection. This created a concern that we might be introducing the pathogen to the wild populations in the Williams Fork River. While the typical concern is pathogen transfer from wild fish to hatchery brood stocks when bringing fish to a hatchery, our objective was different. We wanted to confirm the pathogen's presence in the Williams Fork River system to ensure we were not introducing new pathogen to the area when stocking these potentially infected fish. While *R. salmoninarum* is known to be widespread throughout Colorado's wild Brook Trout populations without obvious negative effects (Kowalski et al. 2017; Firestone et al. *In progress*), we had no evidence of its presence in the specific Bobtail and Steelman Creeks. Therefore, our study aimed to confirm whether the current Brook Trout population was already infected with *R. salmoninarum* before introducing YY male Brook Trout from a potentially positive hatchery source.

1.2 Methods

Brook Trout and Cutthroat Trout were sampled from two locations: Steelman Creek and Bobtail Creek, with the objective of determining the prevalence of *Renibacterium salmoninarum* before the introduction of YY Brook Trout. Kidney, liver, and spleen tissues were collected from each fish through an abdominal incision. Tissues were placed into a whirl-pak-bag and immediately homogenized together to create a single, composite tissue sample. All samples were collected and analyzed in a mobile laboratory in the field under a disinfected PCR hood. Samples were frozen on dry ice immediately and were transported to the laboratory where they were stored at -20°C until sample processing.

1.2.1 Quantitative polymerase chain reaction (qPCR) analysis

For DNA extraction, all tissue samples were thawed and homogenized. Two replicates of approximately 0.25 g of the homogenized tissue sample were prepared for DNA extraction. DNA extractions were completed using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA). To improve DNA yield, the AE buffer volume was increased to 400 µL during all DNA extraction protocols to ensure proper rinsing of filters (Elliott et al. 2013).

Quantitative PCR assays were performed on an ABI StepOnePlus System (Applied Biosystems, Foster City, CA, USA) to detect a 69-base pair DNA segment of the *msa* gene specific to *R. salmoninarum* (Chase et al. 2006). Each reaction consisted of a final volume of 5 µL of extracted DNA template combined with TaqMan Gene Expression Master Mix (ThermoFisher, Waltham, MA, USA) and a predetermined primer and probe set. The primer sequences were RS 1238 Forward (5′-GTGACCAACACCCAGATATCCA-3′) and RS 1307 Reverse (5′-TCGCCAGACCACCATTTACC-3′) with a probe with an MGBNFQ quencher (RS 1262 5′-CACCAGATGGAGCAAC-3′; Chase et al. 2006). DNase-free water was used as a no-template control on each plate. The qPCR cycle threshold (Cq) values were adjusted based on a previously developed standard curve with a cutoff value of 37.75 (Firestone et al. 2025).

1.2.2 Enzyme-linked immunosorbent assay (ELISA)

A double-sandwich ELISA was used to detect soluble antigens of *R. salmoninarum* (Jansson et al. 1996, Elliott et al. 2014). Kidney tissues were prepared to a 1:4 (w/v) dilution in PBS-T20 (phosphate-buffered saline pH 7.4, 0.05% (v/v) Tween-20, and 0.01% (w/v) thimerosal), homogenized, heated at 100°C for 15 minutes, and then stored at −20°C until screening. Affinity-purified *R. salmoninarum*-goat antibodies (Kirkegaard and Perry Laboratories) were used as the coating antibody, and horseradish-peroxidase labeled *R. salmoninarum* goat antibodies served as the antibody conjugate (Pascho et al. 1991).

A control microplate was used for each reaction, containing replicates of cell culture water, HRP-conjugate controls, substrate-chromogen controls, and Brook Trout tissue serving as negative controls. Four concentrations of *R. salmoninarum* positive controls (KPL; 1:100, 1:1000, 1:2000, 1:5000) were also processed in replicates for each reaction. Colorimetric development in each sample was measured at 405 nm with a plate reader. Two cutoff values were used to determine positive samples: one based on published cutoffs (Method 1; Low 0.074-0.199, Mid 0.200-0.999, and High 1.00 +; Elliott et al. 2013; Table 1.1) and a second based on the negative control cutoff (Method 2; Table 1.1), which was calculated as the average OD of the negative control plus 2 standard deviations (ODavg+2∗SD).

Table 1 Optical density values for antigen levels from ELISA based on Method 1 (published cutoff) and Method 2 (Avg OD value of negative control (NC) + 2xStd.dev)

Antigen Level	Method 1	Method 2
Negative	< 0.073	Below NC Threshold
Low	0.074 – 0.199	Above NC Threshold and below 0.10
Mid	0.200 – 0.999	Above 1.00 and below 1/5000 positive control OD Value
High	1.00 +	Above 1/5000 positive control

1.3 Results

1.3.1 Steelman Creek results

A total of 57 fish were collected from Steelman Creek, including 47 Brook Trout and 10 Cutthroat Trout. The qPCR analysis revealed a low prevalence of *R. salmoninarum*, with only 8.8% of the fish (5/57) testing positive from the composite tissue samples. The positive detections included four Brook Trout and one Cutthroat Trout. Overall, the bacterial loads detected by qPCR remained low, with all positive samples having fewer than one bacterial cell per gram of tissue.

The ELISA assay detected a much higher prevalence of *R. salmoninarum* antigens, with results varying depending on the cutoff method used. Using published cutoff values (Method 1), the prevalence was 93% in Brook Trout (44/47) and 100% in Cutthroat Trout (10/10). The majority of positive detections in both species were at the "Low" antigen level (OD values between 0.074-0.199), accounting for 85.1% of Brook Trout and 70% of Cutthroat Trout (Table 1.2).

Using the negative control cutoff (Method 2), which had a threshold of 0.084, the prevalence was 70.2% in Brook Trout (33/47) and 100% in Cutthroat Trout (10/10). Under this method, a greater proportion of fish were classified with "Mid" antigen levels (OD values above 0.10), with 38.3% of Brook Trout and 100% of Cutthroat Trout falling into this category (Table 1.2).

Table 1.2 ELISA results for Brook Trout and Cutthroat Trout tested for *Renibacterium salmoninarum* at Steelman Creek with values for Method 1 and Method 2 where the negative control threshold for method 2 was OD: 0.084.

Species	Antigen Level	Method 1	Method 2
Brook Trout	Negative	6.4% (3/47)	29.8% (14.47)
	Low	85.1% (40/47)	31.9% (15/47)
	Mid	8.5% (4/7)	38.3% (18/47)
	High	0	0
Cutthroat Trout	Negative	0	0
	Low	70.0% (7/10)	0
	Mid	30.0% (3/10)	100% (10/10)
	High	0	0

1.3.2 Bobtail Creek results

A total of 60 Brook Trout were collected from Bobtail Creek. Similar to Steelman Creek, the qPCR assay showed a low prevalence of *R. salmoninarum*, with only 11.7% of the fish (7/60) testing positive. All positive samples had low bacterial loads (<1 bacterial cell per gram of tissue).

The ELISA results for Bobtail Creek also showed higher prevalence rates than qPCR. Method 1 (published cutoff) detected a 100% antigen prevalence, with 75% (45/60) of fish having "Low" antigen levels, 23.3% (14/60) at "Mid" levels, and 1.7% (1/60) at "High" levels. Using the negative control cutoff (Method 2), the overall prevalence was 93.3% (56/60). Under this

method, the distribution of antigen levels was notably different, with 75% (45/60) of the fish classified at "Mid" antigen levels and 3.3% (3/60) at "High" levels.

The difference between the two ELISA cutoff methods highlights the importance of the threshold value in determining prevalence and antigen levels. The negative control cutoff (Method 2) generally led to lower prevalence rates but higher proportions of fish with "Mid" to "High" antigen levels compared to the published cutoff (Method 1).

Table 1.3 ELISA results for Brook Trout and Cutthroat Trout tested for *Renibacterium salmoninarum* at Steelman Creek with values for Method 1 and Method 2 where the negative control threshold for method 2 was OD: 0.084.

Species	Antigen Level	Method 1	Method 2
Brook Trout	Negative	6.4% (3/47)	29.8% (14.47)
	Low	85.1% (40/47)	31.9% (15/47)
	Mid	8.5% (4/7)	38.3% (18/47)
	High	0	0
Cutthroat Trout	Negative	0	0
	Low	70.0% (7/10)	0
	Mid	30.0% (3/10)	100% (10/10)
	High	0	0

In both creek systems, the ELISA assay consistently detected a higher prevalence of *R. salmoninarum* than the qPCR assay. While qPCR results indicated that bacterial loads were low in all positive fish, the high prevalence of antigens detected by ELISA suggests the pathogen is widespread in both populations.

1.4 Discussion

Our finding that *Renibacterium salmoninarum* infections were already established in both the Brook Trout and Cutthroat Trout populations mitigated the concern regarding the introduction of *R. salmoninarum* into the Bobtail and Steelman Creeks via hatchery-sourced YY Brook Trout. Our results showed high antigen prevalence via ELISA aligning with previous research indicating *R. salmoninarum* is far more widespread in wild trout populations than previously assumed (Kowalski et al. 2017), but its presence does not necessarily translate to adverse population-level effects (Firestone et al. *In progress*). While the hatchery-sourced fish were from a known positive source, the risk of widespread disease transmission from stocking is considered low and unlikely to significantly impact the existing population (Riepe et al. 2021, Riepe 2022, Riepe et al. 2022). For instance, even in hatchery lots with high percentages of low-level infections among a brood stock, the average proportion of infected progeny from individual spawning pairs is low (1-21%). Similarly, horizontal transmission was also very low (1%). Additionally, clinical signs of bacterial kidney disease are in wild populations and previous research has found no significant differences in annual mortality across sites with infected Brook Trout, suggesting that *R. salmoninarum* is not causing major population-level effects. Therefore, based on the established prevalence of *R. salmoninarum* in the recipient waters and the low

transmission risk from hatchery-reared fish, we concluded that there was no concern of stocking potentially *R. salmoninarum*-infected YY Brook Trout into Bobtail and Steelman Creeks.

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Chemical Avoidance of Magnesium Chloride by Brown Trout *Salmo Trutta*

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2.1 Abstract

Sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) are used as road de-icers. Streams and rivers adjacent to roads see spikes in conductivity and chloride after snow melt. In many locations fish have been lost or recruitment is limited. Fish are extremely tolerant to chloride in laboratory experiments but the majority of these studies examined only mortality. Chemical avoidance behavior is a possible explanation for a loss of biota. Upstream passage is often poor for fish in Colorado due to barriers and high gradient slopes. Chemical avoidance behavior leads to permanent loss of fish in this situation. To examine if drift is a cause of fish extirpation, salmonids were introduced to a gradient of salt concentrations in a circular chamber. Videos of school behavior were analyzed to determine what concentration can elicit an avoidance behavior.

2.2 Introduction

Government agencies and municipalities are tasked with ensuring public safety on highways while preserving environmental health. This is especially difficult in Colorado, where steep mountain passes and wind swept plains experience frequent snow and ice storms from October to May. De-icing chemicals like sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) have been regularly applied to roads to mitigate winter conditions in the United States and Canada since the 1940s (Evans and Frick 2001). Application of chloride-based de-icers have reduced automotive collisions by up to 78-87% (Hanbali and Kümmel 1992). Colloquially called "rock salt," sodium chloride is typically applied to roads as a solid where the dissociating salt ions cause water to freeze at a lower temperature preventing buildup of ice on roadways (Nazari et al. 2015). Sodium chloride has historically been the most common and economical chloride based de-icer (Hintz et al. 2022). Magnesium chloride is common when application of a liquid de-icer is preferred. Magnesium chloride and calcium chloride ($CaCl_2$) are gaining popularity because they might be less harmful to the environment. Given rising populations in the USA, application rates of NaCl have increased significantly from 164,000 tons in 1940 (Galella et al. 2023) to 22.7 million tons in 2015 (Bolen 2016). Application rates are only expected to continue increasing as human populations continue to grow (Corsi et al. 2010).

Anthropogenic sources of chloride contamination in freshwater include mining, agriculture, and industry. However, within the mountain west of the USA, road salts applied as de-icers are a leading cause of surface water salinization (Vander Laan et al. 2013; Cañedo-Argüelles et al. 2014; Kaushal et al. 2018). The degree to which salinization occurs from road salt inputs is often correlated to increased urbanization and impervious surface cover with more contamination in locations closer to roads (Williams et al. 1999; Evans and Frick 2001; Kelly et al. 2018; Moore et al. 2020). An outlier might be Colorado where highways traverse rural mountain ranges. Colorado's mountain motorways typically parallel the sinusoidal curves of streams and rivers. During rain and snowmelt events de-icing chemicals enter adjacent streams resulting in elevated pulses of salinity, specifically as chloride ions (Nazari et al. 2015). Ground water, soil, and the unsaturated zone can subsequently act as reservoirs of chloride releasing variable loads of ions into surface waters over time (Kincaid and Findlay 2006; Lax and Peterson 2009; Cooper et al. 2014). Aquifers can accumulate chloride and release it to surface waters in unpredictable ways

(Bester et al. 2006; Corsi et al. 2015). Efforts to predict chloride concentrations and implement management practices may become more difficult (Bester et al. 2006).

In 1988, the United States Environmental Protection Agency (EPA) developed acute (860 mg/L) and chronic (230 mg/L) aquatic life criteria for chloride (USEPA 1988). Canada set similar criteria (acute: 640 mg/L; chronic: 120 mg/L; CCME 1999). Studies throughout North America have confirmed that chloride concentrations in surface waters can far exceed chloride aquatic life criteria during pulses (Bester et al. 2006; Allert et al. 2012; Kelly et al. 2018). Sublethal effects across trophic levels of aquatic and semi-aquatic organisms have been observed in riparian vegetation, macrophytes, zooplankton, amphibians, invertebrates, and fishes (Sanzo and Hecnar 2005; Goodrich et al. 2008; Elphick et al. 2011; Kotalik et al. 2017; Coldsnow et al. 2023; Rogalski et al. 2024). Minor alterations in behavior and physiology can induce shifts in community composition, ultimately threatening ecosystem function (Fleeger 2020).

Salmonids fill the ecological and economic niche as apex aqueous predator in Colorado's mountains and are the primary sport fish in high gradient mountain streams and alpine lakes. Native salmonids include only the numerous strains of Cutthroat Trout *Oncorhynchus clarkii* (Metcalf et al. 2012; Bestgen et al. 2019) and Mountain Whitefish *Prosopium williamsoni*. Yellowfin Cutthroat Trout (AKA yellow lineage; Metcalf et al. 2012) went extinct in the late 1800s due to mining pollution and overharvesting in the headwaters of the Arkansas River (Leadville, Lake Country, CO, USA). Extensive effluent treatment and habitat restoration efforts on the Arkansas River (Clements et al. 2010) were unable to re-establish any Cutthroat strain. These restorations did allow Brown Trout *Salmo trutta* to survive in the once fishless waters.

Brown Trout introduction in the 1890s is sometimes viewed as a notorious event as Brown Trout compete with native Cutthroat populations (Wang and White 1994). In many polluted systems, these are the only species that can survive. Trout species fill the role of apex predators in their community (Perälä et al. 2021). Without apex predators Colorado's lotic ecosystems might see trophic cascades, algal phase shifts, a collapse of invertebrates and/or a loss of ecosystem services. Brown Trout and Rainbow Trout *Oncorhynchus mykiss* are non-native salmonids of economic importance in Colorado. The stocking of popular angling fish and culturing of angling opportunities adds $2 billion annually to the state's economy. Brown Trout and Rainbow Trout are able to reproduce and grow to trophy size in waters too warm or too polluted for our native species.

Water quality standards in the state aim to protect all salmonid species. Rainbow Trout have an LC_{50} that ranged from 6,030 - 7,461 mg/L Cl^- after a 96 hour exposure (Spehar 1987; Elphick et al. 2011). When exposed to potassium chloride (KCl), rainbow trout had a 7-day LC_{50} range of 1,586 - 2,828 mg/L and Brook Trout *Salvelinus fontinalis* had a 7-day LC_{50} range of 2,732 - 3,007 mg/L (Lazorchak and Smith 2007). Less is known about the toxicity of chemical effluents on Cutthroat Trout (Coleman 2007), but it is suspected that they are more sensitive to chemical contaminants than Rainbow Trout (Sappington et al. 2001). Fathead minnows *Pimephales promelas* had an acute LC_{50} of 4,079 mg/L Cl^- (Elphick et al. 2011). For mosquitofish *Gambusia holbrooki* the 96 hour LC_{50} was 11,540 mg/L Cl^- (Newman and Aplin 1992). One study used conductivity as a proxy for chloride concentration and found that Pumpkinseed *Lepomis gibbosus* juveniles had a 96 hour LC_{50} of 20,300 µS/cm (Venâncio et al. 2019). These thresholds are significantly higher than observed in nature.

Colorado streams are high gradient, especially near mountain passes where road salt is applied regularly. Even in unaltered watersheds, steep grades of Colorado topography make upstream passage difficult for fish. Anthropogenic barriers such as culverts, dams, and water diversions accommodate downstream migration but impede the upstream return of fish. Chemical avoidance behavior in Colorado's lotic system would result in downstream loss of fish. This could manifest into extirpation of fish species from a watershed if upstream passage is limited. Downstream chemical avoidance of any organisms in a lotic ecosystem is an example of "drift." Drift behavior in fish is rewarded by possible access to improved habitat or escape from unfavorable environmental conditions (Lechner et al. 2016). Benthic macroinvertebrates drift when seeking out new food sources or avoiding predators. They also drift in response to elevated specific conductance (Clements and Kotalik 2016) and other water quality stress. Insect nymphs that succumb to drift have the advantage of aerial dispersal and recolonization after emergence.

Avoidance behaviors in fish are one of the most sensitive sublethal endpoints that can be measured in fish (Hansen et al. 1999). The underlying "proximate" mechanism that triggers avoidance/drift in fish is unknown, but physiological changes in the olfactory cells might contribute. Fish can smell molecules in the water that cue feeding behaviors, reproductive behaviors, and predator avoidance (Yue et al. 2015). Environmental contaminants can damage the olfactory cells altering their physiological ability to sense environmental cues (Kasumyan 2001). Contaminants can also interfere with environmental cues (Yue et al. 2015). Both instances prevent an individual's ability to formulate the correct behavioral response, sacrificing their fitness (Jacquin et al. 2020) and altering community dynamics (Sovova et al. 2014). The "ultimate" benefit of drift behavior is escape from unsuitable environment before loss of life.

Measuring chemical avoidance behavior as an effect in chloride toxicity experiments could reconcile the discrepancy between the high concentrations able to cause mortality in the laboratory and the low concentrations observed at biomonitoring sites where fish populations are reduced. To test if chemical avoidance of chloride could manifest into extirpation of salmonids, we placed Brown Trout in a circular chamber that creates a gradient of toxicant. High resolution cameras were used to record fish behavior. Fish behaviors and occupancy (amount of time fish spend in a location) was analyzed to document chemical avoidance across a gradient of chloride.

2.3 Methods

2.3.1 Test Organisms

Brown trout were obtained from a Bellvue State Fish Hatchery (Bellvue, CO, USA) and were originally spawned from North Delaney Butte Lake (Walden, CO, USA. Oct 2024). No ectoparasite treatments or medication (i.e. chlorine, chloramine, formalin or salt) was used for at least six months prior to experiments. Organisms were held in a large (180 x 45 x 40cm) insulated tank receiving flow-through dechlorinated municipal tap water (Fort Collins, CO, USA) at moderate hardness (47-55 mg/l CaCO4 equivalents). Organisms were maintained at minimum growth feed rate (2-3% body weight/day). Wide spectrum LED lamps produced 16:8 light-dark cycles.

Twenty four individuals of 53 mm standard length (SD=5.6 mm) were selected and randomly assigned to three holding tanks (15 x 20 x 30cm glass aquaria) to provide three groups of eight

fish. Each group was used in control and treatment trials as described below then measured and euthanized (pH buffered MS-222).

2.3.2 Study Design

An annular temperature preference chamber based on Myrick et al. (2004), constructed by Jordan Anderson and Steven Brinkman in 2010 and used by Cipela et al.(2021), was heavily modified to produce a gradient of toxicant, monitor and sample water in each segment, and record high definition video of fish behavior and location. In short, the system is a circular or annular (Figure 2.1) shaped tank that restricts fish to one circular annulus 7 cm deep and 10 cm wide. Fish occupy only the swimming annulus, which lacks corners. This avoids loafing or taking shelter in corners of traditional rectangular aquaria. The distribution annulus receives flow-through water from a suspended ring shaped diluter system which provides a calibrated flow and precise concentration to each of the 32 segmented chambers. Water travels through perforated walls of the swimming annulus, transverses across the swimming annulus, and past the perforated walls of the effluent annulus where the 32 segmented chambers empty into the drainage annulus at equal rates.

The back lines on the floor and walls of the swimming annulus demark the 32 numbered segments occupied by fish with 1 and 32 receiving the highest concentration and 16 receiving the lowest or control concentration of toxicant. Four high definition cameras (4K 3840x2160 resolution at 15fps. GoPro Hero 3+ Black Edition. GoPro, Inc. San Mateo, CA, USA) recorded fish location and behavior. Videos were synchronized using multiple atomic clocks visible from all camera angles. Conductivity (an established surrogate for some toxicants) and temperature was continuously recorded with conductivity probes (Groline HI98331. Hanna Instruments, Inc. Woonsocket, RI, USA) visible in each camera. Each camera was monitored by HDMI or WiFi connections to screens to ensure full visibility of fish, clocks, and probes. Six evenly spaced incandescent 60 W (650 lumen) large diameter white frosted globe lights (Product 03107, Westinghouse Lighting, Philadelphia, PA, USA) were hung 10 cm above a white bed sheet that was 5cm above the level of the cameras. This provided diffuse illumination through the cloth and prevented glare. Dark curtains surrounding the system prevented fish from being started by technician movement. Thirty two small capillary tubes with Luer-Lok fittings allowed water samples to be drawn from each of the 32 segments. Head tanks controlled by pipe stands and float valves received dechlorinated municipal tap water calibrated to the same (+/- 0.2ºC) temperature as fish holding and rearing tanks. Metered flows were delivered to diluter rings with the bottom ring receiving chloride stock solution by a calibrated peristaltic pump. Groups of fish were video recorded in a control (no $MgCl_2$) and a treatment (gradient of $MgCl_2$) trial of 30 minutes. Videos of each trial were synchronized by the atomic clock time stamps and joined into one video for behavior analysis by trained technicians.

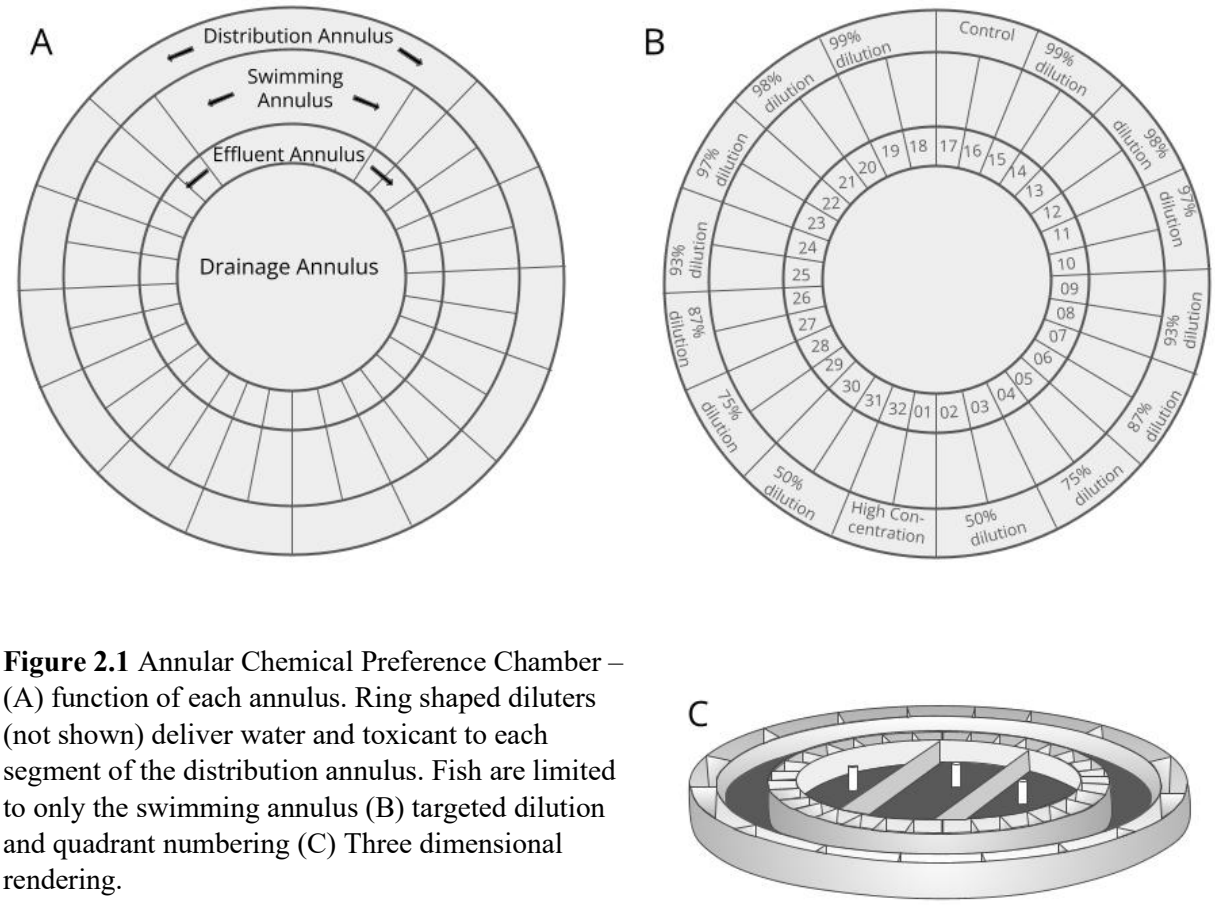

Figure 2.1 Annular Chemical Preference Chamber – (A) function of each annulus. Ring shaped diluters (not shown) deliver water and toxicant to each segment of the distribution annulus. Fish are limited to only the swimming annulus (B) targeted dilution and quadrant numbering (C) Three dimensional rendering.

2.3.3 Fish Behavior

Fish kill events are rare to observe in real time, often discovered days after the event, or are captured only as a drop in population in annual biomonitoring. Colorado Parks and Wildlife has had staff on-site for limited active toxic events (*e.g.* pH, acid mine drainage, aqueous metals). Additionally the agency has decades of observations of piscicide application to remove nuisance and non-native fish (*e.g.* Fintrol/antimycin, Prenfish, Rotenone, CTF Legumine). Observations from these events were summarized into common chemical avoidance behaviors:

1- Pause: an initial stop in movement and travel.
2- Directional Orientation: minimal body movement or change of direction, position or orientation but visual and perhaps olfactory sensing of threats. Often co-occurring with shoaling or looking to other fish for confirmation.
3- Directional Probing: body orientation or movement in a range of directions to determine where water has or does not have the offending scent or irritation. This typically defines the next direction of travel or avoidance behavior.
4-Shoaling: grouping with other fish of the same species and size class, a common stress response.
5- Directional Change: change of path from the direction originally traveled by the

individual or school. Typically presents as a 180 degree alteration (reversal) of course in narrow lotic systems.
6- Bolting: increase in velocity or pace when in the presence of the toxicant in an innate effort to "pass through" the offending plume.
7- Nosing: deliberate efforts to explore ground water intrusion (fresh water entering streams in the hyporheic zone) in substrate, explore small tributaries, and explore or jump into small falls and trickles.

Rubrics (ethograms) were used to categorize (*e.g.* yes vs no) or rate severity (*e.g.* 0, 1, 2, 3, 4, 5) of fish behavior in the experimental apparatus. When possible ethograms were avoided and measurements of occupancy (location), direction, speed, changes in orientation, and distance to other fish were used as less subjective measures. Random number generators defined time points in all videos for behavior assessments. The following were assessed:

Occupancy: at randomly assigned time points in the videos location of fish in the system was recorded along with conductivity. We predicted occupancy would be evenly distributed across all quadrants in control trials (no-chloride added) but fish would avoid the high chloride side of the annular chamber during exposure trials (chloride gradient present). We further predicted this avoidance trend would have increased occupancy in the quadrants or conductivity levels in which fish pause and change direction of travel.

Direction of Travel: during the 10 seconds bracketing each randomly selected time point any direction changes of each fish were recorded along with the quadrant and conductivity. We predicted direction changes away from the high toxicant concentration would out number direction changes towards the high concentration quadrants in exposure trials (t-test) but no difference would be observed during control (no-toxicant) trials.

Bolting: during the 10 seconds bracketing each randomly selected time point was assessed for fish passing the three lowest concentration quadrants and the highest three quadrants. Velocity was calculated using elapsed time and distance (determined by lines demarking quadrants). We predicted velocities of the high concentration would be faster than the control (t-test) in exposure trials but that velocities would be indistinguishable in control (no chloride) trials.

Shoaling: at each randomly selected point in each video shoaling was assessed by ethogram and also by enumerating the number of fish with two other fish within two body length distance. We predicted the proportion of fish near other fish to be greater in exposure (chloride) trials than in control (no-chloride) trials (t-test).

Directional Probing, Nosing, Directional Orientation: during the 5 seconds bracketing randomly assigned time these behaviors were assessed by ethogram.

2.3.4 Water Chemistry

Conductivity was monitored by 16 calibrated conductivity probes (see above). Water samples from each quadrant were sampled during each trial. Inorganic elements were assessed by ICP-OES. Chloride was assessed by EPA method 325.2 using a flow injected colorimeter (Lachat QuickChem 8500 series 2. Hach. Loveland CO USA. Method 10-117-07-1-H).

2.2.5 Statistical Analysis

Video analysis for all behaviors aside from occupancy is on-going.

Occupancy- Data was analyzed using a Bayesian occupancy model using Just Another Gibbs Sampler (JAGS) within program R (version 4.4.3; R Core Team 2025). Our model calculated the probability that a fish would occupy each of the 32 quadrants given the quadrant's water conductivity, hereafter referred to as occupancy. Our data consisted of, quadrant number, trial number, average conductivity of each quadrant among trials, the sum of the total number of fish within a quadrant per visit, and total number of visits per quadrant (117) per trial. To speed up model convergence and remove units, the predictor values of average conductivity were scaled. Inferences were based on the derived quantities of the mean occupancy within a quadrant given water conductivity, the difference between occupancy of a control treatment compared to a chloride treatment, and the 95% credible interval overlap.

2.4 Results

2.4.1 Behavior

Analysis of videos is pending for most behavior endpoints. However, incidental observations suggest a clear avoidance of chloride. Brown Trout often shoaled within both control treatments and chloride treatments, but with differing movement patterns within shoals. For example, in control treatments, Brown Trout swam in complete circles throughout the annular preference chamber, sometimes switching directions or exploring the new environment. Alternatively, in chloride treatments, they swam in what appeared to be "U" shaped patterns, turning around in quadrants with elevated chloride concentration (Figure 2.2).

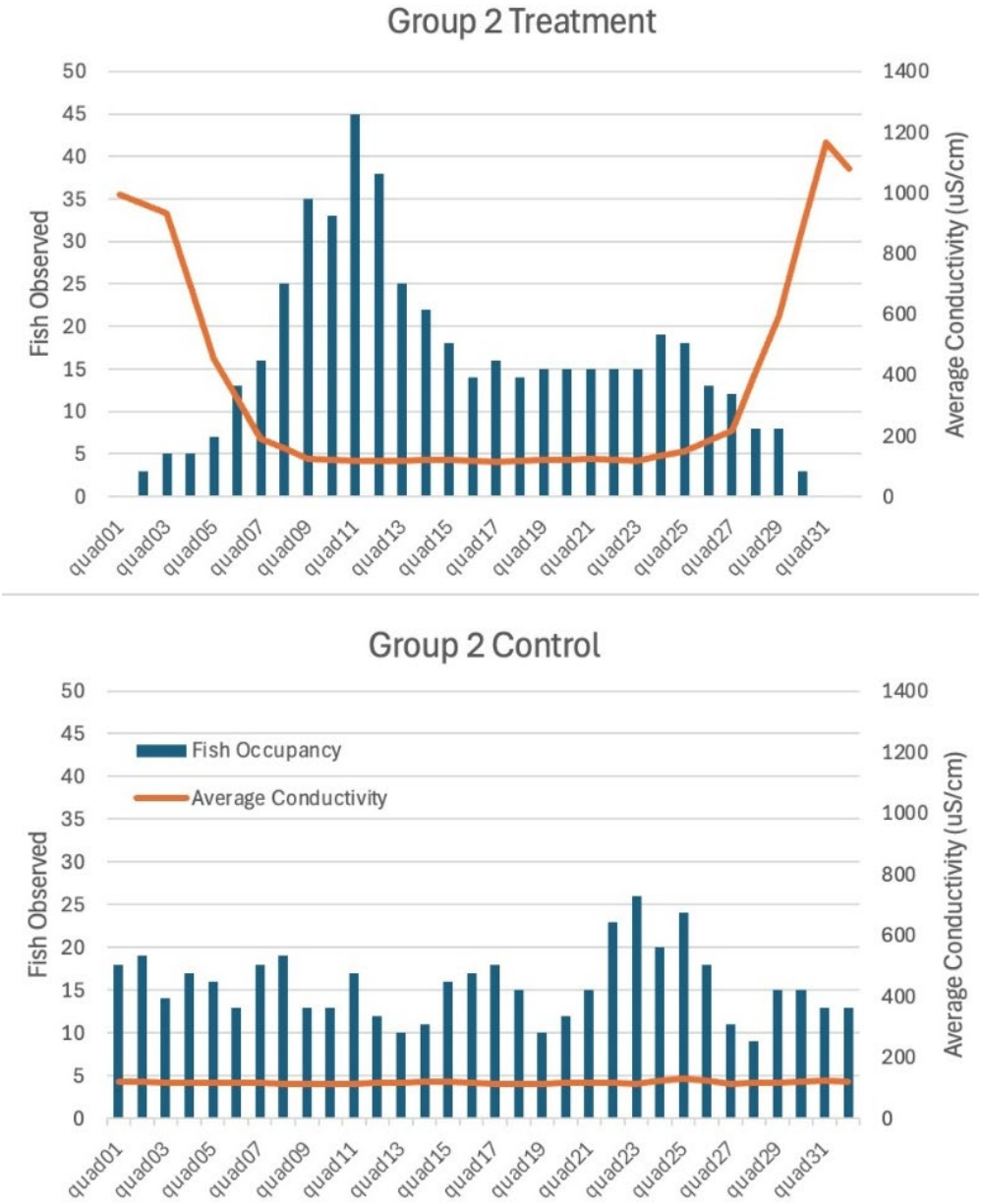

Figure 2.2 Observed fish presence at randomized time points in a control and treatment trial. Results from one of three groups of fish presented here as an example. High $MgCl_2$ was assigned to quadrants 1 and 32 for only the treatment trial. Conductivity shows background (no $MgCl_2$) readings for quadrants 15 to 19 of the treatment trial and all quadrants of the control trial. No fish were observed in quadrants 1, 31, and 32 when $MgCl_2$ was present. During treatment trials, increased observations of fish near quadrants 9 and 25 was likely "Pause" and "Direction Change" behaviors. Fish occupied all quadrants equally during control trials.

2.4.2 Occupancy

The preliminary model converged based on our trace plots showing good mixture and R_{hat} values of the parameters of interest all being less than 1.03. Posterior predictive checks (Bayesian *p*-values) were all greater than 0.1 and less than 0.9 (mean *p*-value = 0.54; standard deviation *p*-value = 0.32; Hobbs and Hooten 2015). The occupancy for the control treatments ranged between 0.15 - 0.34 and the occupancy for the chloride treatments ranged between 0.056 - 0.47 (Figure 2.3). A negative trend associated with chloride concentration and occupancy was observed; as conductivity increases the probability of occupancy decreases. Comparing the control treatment and chloride treatment, occupancy resulted in a statistically significant difference. Quadrants with high conductivity (quadrants 30, 31, 32, 1, 2, 3) show significantly lower occupancy compared to control treatments within the same quadrants (Figure 2.4).

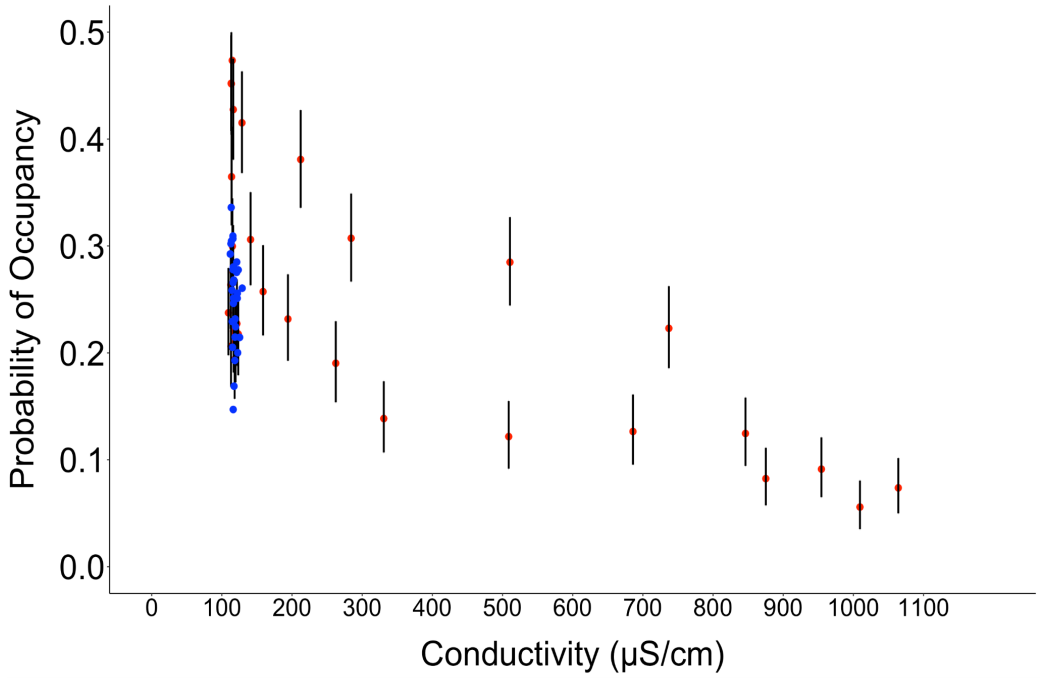

Figure 2.3 The probability of fish occupancy in control trials (No $MgCl_2$. blue dot) and treatments trials ($MgCl_2$ gradient. red dot) for each quadrant as a function of conductivity. Whiskers represent 95% credible intervals. As conductivity increases, the probability of occupancy within a quadrant decreases.

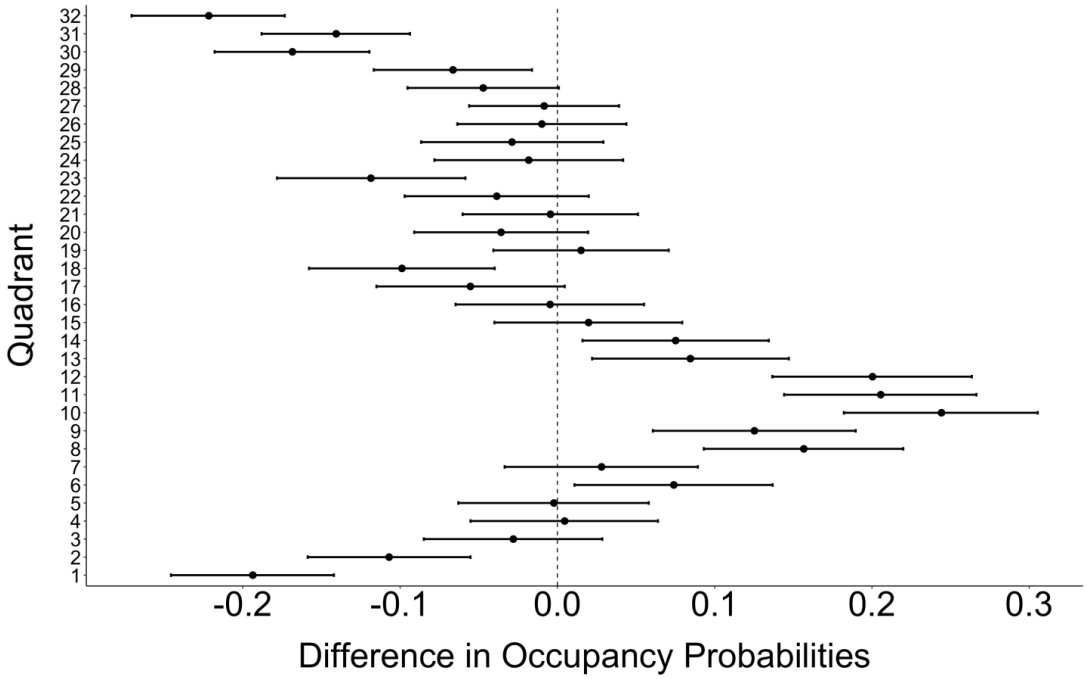

Figure 2.4. The difference in modeled occupancy probabilities between treatment trials ($MgCl_2$ gradient) and control trials (no $MgCl_2$) for each quadrant. Negative values denote that the probability of occupancy in a quadrant is lower in treatment trials than in control trials. Positive values denote that the probability of occupancy in a quadrant is higher in treatment trials than control trials. Whiskers represent 95% credible intervals. Values with credible intervals that do not overlap zero are significantly different between the treatment and control trials.

2.5 Discussion

Our results showed that as conductivity increases occupancy decreases. As predicted, a slight increase in occupancy was observed near quadrants 10 and 25. We suspect this is where chloride nears a threshold causing alarm, olfactory cue or irritation. Further analysis will show if these locations subsequently initiate a directional change (180 degree change from the intended direction) or a bolting behavior. This shows salmonids likely avoid chloride. In nature, this would result in local loss of individuals to drift. If upstream passage is limited, this population could easily be extirpated, especially if the local basin offers no refuge in unpolluted tributaries. Improved occupancy models are on-going.

Observations of sensitive insect species (*e.g.* mayflies) where fish have been extirpated are quite likely in a pulse or seasonal exposure regime as observed near highways. Insect species emerge as adults and lay eggs in upstream waters. Colorado fish species cannot recolonize aerially. Ensuring upstream fish passage near highways has ecotoxicological implications in addition to population genetics and migration implications.

Future research will examine sensitivity of other fish species and age classes. Sodium chloride may be compared to magnesium chloride and de-icers that contain anti-corrosion inhibitors (not-present in this study). Aqueous metals, pH, diesel, gasoline, tire wear chemicals, and other analytes will be considered as requested by managers.

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Dr. Tawni B. R. Firestone

3.1 Introduction

Freshwater ecosystems are vital habitats for a wide variety of aquatic organisms. The health of these systems is linked to water quality, which directly influences survival and reproductive success of aquatic organisms. A significant and growing threat to these ecosystems is "Freshwater Salinization Syndrome" (FSS), which is progressively altering critical habitats across North America (Kaushal et al. 2018). FSS is characterized by increased concentrations of salts, with the widespread application of road salts during winter being the primary anthropogenic driver. High-elevation streams in close proximity to busy roadways are particularly vulnerable to elevated salt concentrations. While the use of road salts is crucial for public safety and accident reduction, it poses a significant environmental cost. Previous research shows salt causes corrosion to infrastructure, contaminating ground water and surface water sources, causing harm to freshwater ecosystems and wildlife, and degrading soil quality (USEPA 2020, Stranko et al. 2013).

The most common deicing agents are inorganic, chloride-based salts such as sodium chloride (NaCl), magnesium chloride ($MgCl_2$), and calcium chloride ($CaCl_2$). These compounds are environmentally persistent, as they do not degrade (Evans and Frick 2001; Corsi et al. 2010). Instead, they are transported into nearby waterways through precipitation and runoff, leading to the long-term accumulation in soils, groundwater, and surface waters (Maltby et al. 1995). This process has resulted in an increase in chloride concentrations in streams, particularly within watersheds containing extensive impervious surfaces like roadways. Data from the U.S. Geological Survey indicate that the quantity of applied road salt has tripled over the last three decades, a trend directly correlated with the large-scale salinization and alteration of freshwater ecosystems (Dugan et al. 2017, Hintz and Relyea 2019).

Elevated salt concentrations affect a broad spectrum of aquatic species, from microbes to fish (Corsi et al. 2010; Hintz and Relyea 2019), leading to reduced biodiversity, decreased population sizes, and the extirpation of native species (Jones et al. 2017, Kaushal et al. 2021). Much of the existing research on effects of road salts has been toxicity testing to determine lethal concentrations. This information is critical for establishing aquatic life standards, such as those recommended by the U.S. Environmental Protection Agency (EPA). For example, the EPA recommends an acute chloride criterion of 860 mg/L and a chronic criterion of 230 mg/L to protect aquatic life. Additionally, the Clean Water Act (CWA) provides the primary regulatory framework for protecting freshwater ecosystems by restricting pollutant discharge (33 U.S.C §1251 et seq. 1972). Under the CWA, the EPA develops and recommends water quality criteria, which are used by states to implement specific water quality standards to protect designated water uses including agriculture, water supply, recreation, and aquatic life. These standards regulate both acute effects (lethal or severe effects within hours to days) and chronic effects (sublethal effects over a longer period).

Mortality toxicity tests provide critical information to understand the worst-case scenario under which the target species would not live after exposure. Lethal concentration is then used to determine the maximum lowest concentrations of salts in waterways that is allowed. Although

endpoint mortality tests do provide critical information for development of aquatic life standards, they lack the ability to make inferences on sublethal effects after exposure. The capacity of fish to cope with stressors is a main component of the normal functions of a fish, because stressors affect the reallocation of energy towards different behavioral mechanisms (Alfonso et al. 2020). The functional definition of stress has been cited as "a condition induced by a stressor that evokes an endocrine response that could be beneficial as well as disadvantageous" (Gorissen and Filk 2016). Responses to stress are not inherently negative, but the associated physiological and behavioral adjustments that fish need to adapt to can cause negative influences, especially when the stress is repeated or other stressors are present (Barton 2002). Thus, it is important to understand how $MgCl_2$ exposures generate a stress response to fish and how added stressors, such as an increase in temperature, also affect the fish. In this study, we measured the level of stress fish are under after $MgCl_2$ exposures by measuring free water-borne cortisol, the stress steroidal hormone.

Despite the EPA's recommendations, many states, including Colorado, lack explicit numeric or narrative regulatory standards for aquatic life for discharges of chloride. A comprehensive understanding of the sources and consequences of increasing salt concentrations is essential for effective environmental management. To address this gap, we conducted a series of toxicity tests on Cutthroat Trout *Oncorhynchus clarkii stomias*. We chose this species because they are a native species in the high-mountain streams in Colorado, a region experiencing significant impacts for road salt runoff (Rust et al. 2024). The exposures were performed on three age classes: 30-day post swim-up (30 d psu), 6-month-old juveniles, and 1-year-old fish and mortality and stress were evaluated after an acute (96 hour) exposure.

3.2 Methods

One-year-old and 30-day post-swim-up (d psu) Cutthroat Trout *Oncorhynchus clarkii stomias* were sourced from the Glenwood Springs Hatchery (Glenwood, CO, USA), while six-month-old juveniles were obtained from the Durango Hatchery (Durango, CO). All fish were transported to the Aquatic Toxicology Laboratory (Fort Collins, CO) and maintained in temperature-controlled troughs at 13°C. They were fed a daily maintenance diet of Bio-Oregon feed equivalent to 3% of their body weight, and their tanks were cleaned every two days throughout the experiment.

Stock solutions were prepared by dissolving analytical reagent-grade magnesium chloride ($MgCl_2$) in deionized water. This stock was delivered via a peristaltic pump at a flow rate of 2.0 mL min^{-1} to a serial diluter. The diluter was used to deliver five nominal concentrations in a 50% dilution series, in addition to a source water control. Salt solutions were delivered to each tank at a flow rate of 40 mL min^{-1}. All tests were conducted in 5-L glass tanks submerged in a 13°C water bath to maintain stable temperatures.

For one-year-old and six-month-old fish, nominal $MgCl_2$ concentrations were 0, 53.75, 105.5, 215, 430, and 860 mg mL^{-1}. Six-month-old fish were also exposed to an additional series of concentrations: 156.25, 312.5, 625, 1250, and 2500 mg mL^{-1}. The one-year-old fish exposure was conducted twice using different densities: a low density (n = 1 fish) and a high density (n = 10 fish). Four replicates for each concentration and densities (one-year-old fish) were included. For the 30 d psu fish, fish were exposed to a narrower range of concentrations (0, 260, 860 mg mL^{-1}), with an increased sample size of 16 fish per concentration.

At the start of each experiment, Cutthroat Trout were distributed one at a time with a hand net into each exposure tank until the desired number of fish was reached. The tanks consisted of 5-L tanks located in a water bath supplied with 13°C water to maintain temperatures. During the exposure, 50 mL water samples were collected four times a day (every six hours) to determine the actual magnesium and chloride concentrations. Additionally, 15 mL of water was collected at every sampling event during the one-year-old fish exposure for cortisol analysis and was frozen at -20°C until use. At the end of each experiment, fish were euthanized, weighed (g), and measured (mm).

3.2.1 Water Quality and Chemical Analysis

Diluters were monitored daily to ensure consistent operation. To confirm actual chloride concentrations, 50 mL water samples were collected from each tank at the start of the 96-hour experiment and every six hours thereafter. To prevent cross-contamination, a new tube and gloves were used for each tank during the sampling process.

Actual chloride concentrations were determined using a flow-injection analysis (FIA) system with a Latchat instrument. The system utilized a mercury-based color reagent dissolved in a solution of methanol, thiocyanate, and nitric acid. Samples were injected into the carrier stream via an 18.5 µL sampling loop. The resulting color reaction was measured by a spectrophotometer at 480 nm. To ensure the accuracy and reliability of the data, each run included stock chloride standards (0-100 mg L^{-1}), duplicates, spiked samples, and internal and external verification standards.

3.2.2 Cortisol Extraction and ELISA

We followed the protocols outlined by Fiebertshauser et al. (2010) for free cortisol extraction from water samples. Water samples were filtered through ethanol-primed C-18 cartridges (Sep-pak, Waters Technology Corporation, Milford, MA) for phase extraction. Cortisol was eluted from the cartridge with two 2 mL washes of 99.5% ethyl acetate and then evaporated under nitrogen gas. Residuals were eluted with EIA buffer as recommended in the ELISA protocol (Cayman Chemical, Ann Arbor, MI).

Each sample was added to a 96-well plate containing 50 µL of sample and 50 µL of cortisol-AChE tracer with dye. An eight-point standard curve was included in each run. After an overnight incubation at 4°C, wells were rinsed with wash buffer, and 200 µL of Ellman's reagent was added to each well. The plate was covered and placed on an orbital shaker for 120 minutes. Following the development of the plate, absorbance was measured at the wavelength of 414 nm using a plate reader to compare to the cortisol standard curve (pg mol^{-1})

3.2.3 Critical Thermal Maximum Tests

Critical thermal maximum (CTMax) trials were performed on 30 d psu and one-year-old Cutthroat Trout from the low-density treatment tanks. We followed the recommendations of Becker and Genoway (1979) for the thermal tests. For each $MgCl_2$ exposure treatment, we aimed to use all fish in the $MgCl_2$ exposure experiment for CTMax trials.

Once the exposure experiment ended, fish were netted and placed individually into a rectangular glass tank with 2 L of water. The starting temperature was the acclimation temperature of 13°C. B-series Love temperature controllers were used to program a temperature increase of +3°C per minute (18°C per hour) for the CTMax trials (Beitinger et al. 2000). Water was heated using aquarium heaters connected to the temperature controllers. Traceable Lollipop thermometers, calibrated before each test, were used to record pre- and post-trial temperatures. Tanks were aerated with atmospheric oxygen, and magnetic stir bars were used to maintain a homogenous temperature. Dissolved oxygen was measured before and after each trial, and water was renewed for each new trial at the starting temperature.

Each trial continued until a sustained loss of equilibrium (LOE) was observed for greater than 10 seconds, LOE, defined as the inability to maintain an upright orientation, has been used as a standard endpoint in previous temperature tolerance studies (Bennett and Beitinger 1997; Selong et al. 2001; Carveth et al. 2006). After each trial, final LOE temperatures were recorded, fish were euthanized with tricaine methanesulfonate (MS-222; Western Chemicals) and their total length (mm) and weight (g) was recorded.

3.3 Results

The water samples, both from the source and before the experiment, had low initial chloride levels. To account for this, we adjusted the water samples results when reporting the $MgCl_2$ exposures. The actual concentrations of chloride in the experiment were on target with the nominal concentrations (Figure 3.1). Throughout all experiments, dissolved oxygen levels remained stable, staying within 2% of saturation.

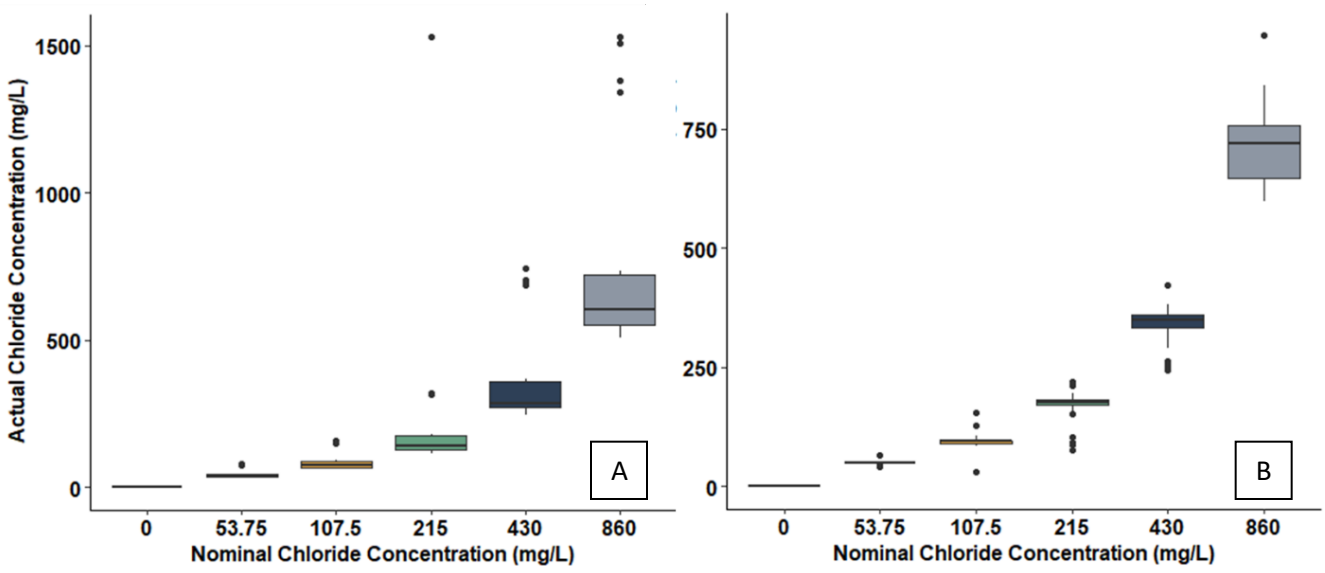

Figure 3.1 Actual chloride concentrations (mg L^{-1}) among the six exposures for high density (A) and low density (B) treatments.

3.3.1 One-year-old Cutthroat Trout

No mortality was observed in any of the exposure treatments, regardless of fish density for the one-year-old Cutthroat Trout.

The CTMax was assessed in the low-density fish groups across eight trials per $MgCl_2$ concentration. Our analysis revealed no significance in thermal tolerance between any of the concentrations ($F_{1.947, 5}$, $p = 0.136$), despite a non-significant drop in the LOE at the lowest concentration of 53.76 mg mL^{-1} (Figure 3.2).

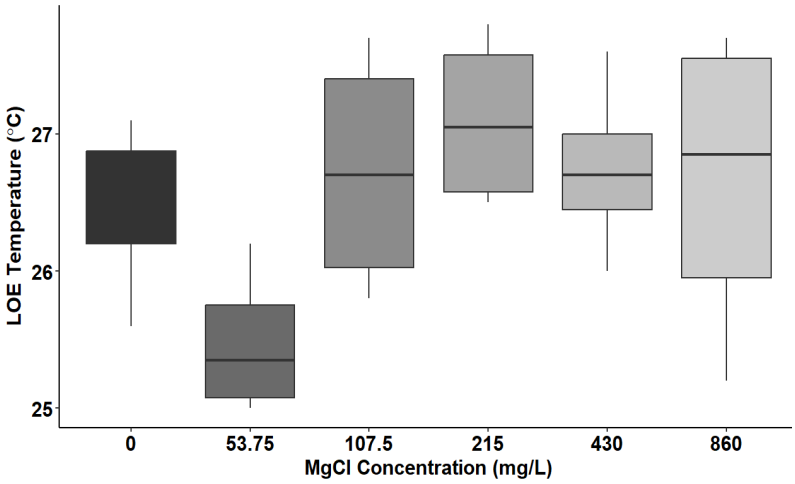

Figure 3.2 Loss of equilibrium (LOE) from critical thermal tolerance tests as a function of magnesium chloride concentrations.

Waterborne cortisol concentrations were not significantly affected by the $MgCl_2$ concentrations, and no significant interaction between density and $MgCl_2$ was detected ($F_{1.87, 5}$, $p = 0.098$). However, cortisol levels were significantly higher in the high-density treatments compared to the low-density treatments ($F_{16.03, 1}$, $p < 0.005$: Table 1, Figure 3.3). These results indicate that fish density, but not $MgCl_2$ exposure had a significant effect of physiological stress as measured by cortisol levels.

Table 3.1 Results based on an ANOVA linear regression models which waterborne-cortisol concentrations (log cortisol pg mL^{-1}) were treated as a function of MgCl2 exposures and density treatments. Outputs are given for each of the models including sum of squares, F-value, and P-value.

Variable	Df	Sum of Squares	*F*-value	*P*-value
$MgCl_2$ Treatment	5	5.84	1.84	0.10
Density	1	9.65	15.19	< 0.05
$MgCl_2$ Treatment * Density	5	1.01	0.32	0.90

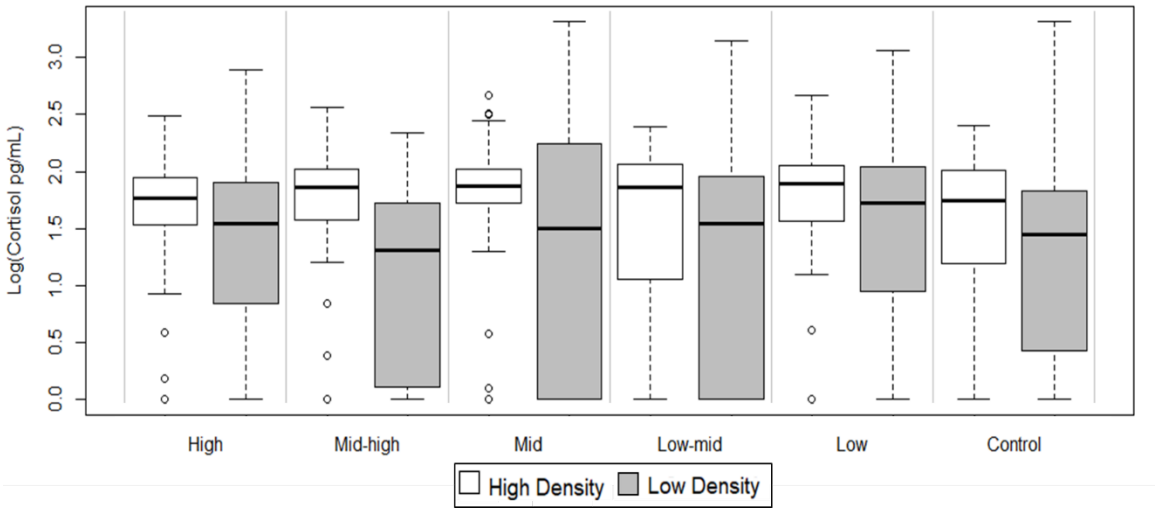

Figure 3.3 Log10 water-borne cortisol concentrations (pg mL^{-1}) collected from high (860 mg L^{-1}), mid-high (430 mg L^{-1}), mid (215 mg L^{-1}), low-mid (107.5 mg L^{-1}), low (53.75 mg L^{-1}), and control (0 mg L^{-1}) $MgCl_2$ exposures from low density treatments (1 fish) and high density treatments (10 fish).

3.3.2 Six-month-old Cutthroat Trout

No mortality was observed when six-month-old fish were exposed to $MgCl_2$ at concentrations ranging from 0 to 1250 mg L^{-1}. However, a single mortality occurred at the highest concentration tested, 2500 mg L^{-1}. We also attempted to measure cortisol in the water. However, no cortisol was detected, likely because the small size of the juvenile fish meant their cortisol excretion was below the detection limits of our assay.

3.3.3 30 d psu Cutthroat Trout

30 d psu Cutthroat Trout showed no mortality at concentrations of 0, 260, or 860 mg L^{-1} $MgCl_2$. However, significant differences were found in thermal tolerance. The fish exposed to the highest concentration (860 mg L^{-1}) exhibited a significant lower tolerance to increased temperatures compared to the control treatment ($F_{5.018,\,2}$, $p = 0.01$). This suggests that while not lethal, exposure to higher salt concentrations may impact the physiological resilience of juvenile fish to other environmental stressors, such as temperature fluctuations.

3.4 Discussion

This study was conducted to investigate the lethal and sublethal effects of $MgCl_2$ on Cutthroat Trout at different life stages. Our research focused on a range of physiological responses, including mortality, cortisol levels, and thermal tolerance (CTMax). We used three distinct age groups; one-year-olds, six-month-olds, and 30 d psu, to determine if sensitivity to $MgCl_2$ exposure varies with age. For one year-old-cutthroat trout, we also examined the potential interaction between $MgCl_2$ concentration and fish density (high: 7 fish, low: 1 fish).

Across all age groups, concentrations, and densities tested, mortality was extremely low, with only a single mortality event occurring in a six-month-old fish at the highest concentration (2500 mg L^{-1}). Due to the lack of significant mortality, we were unable to determine the lethal concentration (LC50) for $MgCl_2$ in Cutthroat Trout at any life stage. This finding indicates that $MgCl_2$ is not acutely toxic to this species at the concentrations tested. Our findings contrast with some studies on other aquatic organisms. For example, Kotalik (2017) observed significant reductions in macroinvertebrate abundance and taxa richness below the chronic chloride water quality standard of 230 mg L^{-1}, and Hassanen et al. (2025) found that chronic exposure led to a decline in growth and histological changes in Nile tilapia. However, our results align with research on the coldwater fish, Rainbow Trout, which have shown no impact on growth with exposures up to 3000 mg L^{-1}.

We assessed physiological stress by measuring water-borne cortisol levels and found a clear distinction between the effects of salt exposure and fish density. For the six-month-old juvenile fish, we were unable to detect cortisol in the water, likely because their small size resulted in cortisol excretion below the detection limits of our assay. In contrast, for the one-year-old fish, $MgCl_2$ concentration had no significant effect on water-borne cortisol levels. This lack of a stress response supports our mortality data, which also indicated that the $MgCl_2$ concentrations tested may not have been high enough to induce a measurable physiological stress response. Cortisol levels were significantly higher in high-density treatments compared to low-density treatments, suggesting that crowding is a greater physiological stressor than the $MgCl_2$ concentrations used in this study. This aligns with the known practice of using salt to reduce stress during fish transport, where concentrations up to 1% can minimize osmoregulatory stress and improve survival during transport (Tacchi et al. 2015). While many studies have shown that organic pollutants can act as endocrine disruptors and cause stress responses, our findings on an inorganic compound like $MgCl_2$ are unique. Our water-borne cortisol results indicate a stress response due to higher fish densities, but they do not support our initial hypothesis that high $MgCl_2$ concentrations would induce a stress response. This highlights the importance of moving beyond mortality tests, which provide critical but limited information for developing aquatic life standards. The capacity of fish to cope with stressors is central to their normal functions, as stress requires the reallocation of energy towards defense mechanisms (Alfonso et al. 2020). As Gorissen and Filk (2016) note, stress responses aren't inherently negative, but the associated physiological and behavioral adjustments can become detrimental, especially when the stress is repeated or multiple stressors are present (Barton 2002).

Fish have specific tolerances to both temperature and salt, but their ability to cope with one stressor may be significantly altered by the other. This is due to the high energetic costs of maintaining internal salt and water balance (osmoregulation) and dealing with thermal stress. When a fish must dedicate more energy to one process, its capacity to handle the other may be compromised (Farias et al. 2024). We assessed thermal tolerance in both one-year-old and 30-day post-swim-up (d psu) fish after exposure to salt concentrations up to 860 mg L^{-1}. Our results suggest an age-dependent effect. The one-year-old fish showed no significant effect of thermal tolerance after exposure to $MgCl_2$, indicating a high resilience. However, the 30 d psu fish, typically known as the most sensitive life stage, demonstrated a significant reduction in thermal tolerance when exposed to the highest concentration of 860 mg L^{-1}. This suggests a sublethal effect in the absence of mortality, underscoring the importance of examining multiple endpoints

beyond survival. This reduced thermal tolerance is likely due to the combined physiological demands of osmotic and thermal stress. Changes in external ion concentration increase the energetic costs of osmoregulation, leaving less energy for coping with rising temperatures (Lockwood et al. 2010; Farias et al. 2024). This has potential management implications, particularly in the context of climate change and altered water chemistry from road salt runoff. The effect seen in the youngest and most vulnerable fish may be concerning for wild populations in increasingly saline and warming waters.

3.5 Conclusion

Our study demonstrates that while $MgCl_2$ is not acutely lethal to Cutthroat Trout, it does exert significant age-dependent sublethal effects. The one-year-old fish were highly resilient, showing no significant impacts on mortality, cortisol, or thermal tolerance. In contrast, the most sensitive life stage, the 30-day post-swim-up fish, exhibited a reduction in thermal tolerance at the highest concentration, even in the absence of mortality. These findings highlight the importance of assessing sublethal endpoints, as mortality alone does not fully capture the physiological stress caused by salt exposure. A key limitation of our study was the inability to detect cortisol in the smaller, younger fish, likely due to low excretion rates. Additionally, our acute exposure design provides only a short-term snapshot of the effects. Therefore, future research should focus on chronic (long-term) exposure studies to evaluate the impact on other crucial parameters like growth and reproductive success. It would also be valuable to test other salt types, such as NaCl, which has been shown to have a negative effect on Rainbow Trout when compared to $MgCl_2$ (Hintz and Relyea 2017). Finally, investigating the combined effects of multiple stressors, such as $MgCl_2$ and emerging contaminants like 6PPD-quinone, would provide a more comprehensive understanding of how freshwater fish populations will cope with the complex challenges of a changing aquatic environment.

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Uveitis, Keratitis, Lateral line Pathologies and Reduced Predator Avoidance in Small Prey Fish *Leuciscidae* after 24hr Exposure to Low Levels of Diesel Fuel Oil

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4.1 Abstract

Petroleum chemicals reach freshwater habitats and can elicit acute toxic effects on ecosystem members. Until recently acute toxicology experiments reported only mortality at 96 hrs, but in nature sublethal effects can manifest into mortality and in lotic environments (streams and rivers) exposure times can be far shorter (<24hrs). Additionally, diesel has been historically studied and regulated as its individual components, but diesel is a mixture of chemicals that could interact synergistically. To model a diesel spill in a lotic environment Plains Minnow *Hybognathus placitus*, Fathead Minnow *Pimephales promelas*, and Flathead Chub *Platygobio gracilis* were exposed to sublethal concentrations of diesel fuel for 24 hours and then paired with control prey and predators Green Sunfish *Lepomis cyanellus*, Yellow Perch *Perca flavescens*, and Creek Chub *Semotilus atromaculatus*. Sublethal endpoints likely to occur in short sub-acute duration (<96hr) that might cause poor predator avoidance were explored. Diesel-exposed Fathead Minnow had significantly decreased neuromast counts likely limiting lateral line function. Corneal opacity was obvious in all exposed fish by sub-gross microscopy. Uveitis and keratitis were present in only exposed fish. These ophthalmic pathologies likely reduce vision and are painful in mammals. Decreased predator avoidance likely arose from sensory and vision impairment. Recovery from ophthalmic pathologies is unlikely in the 48hr or 30d durations pertinent to water quality policy. These sublethal endpoints will be used in future work examining analytes associated with produced waters and fuel spills.

4.2 Introduction

Petroleum spills are frequent anthropogenic disturbances across the world (Schmidt-Etkin 2011, Hettithanthri et al. 2024; Azevedo-Santos et al. 2016; Bacosa et al. 2025). Policies have helped decrease some spill events but they remain a persistent problem due to growing population and energy demands. Although deep sea wells and tanker ship spills capture most media attention, small freshwater spills are equally if not more common (Ivanov et al. 2023; Jiménez Madrid et al. 2015; Mustafa et al. 2024). This is especially true for the Headwater State. Fuels (petrol/gasoline and diesel oils) from highway accidents and crude oil spills from well sites and pipelines enter freshwater ecosystems by accident while produced water discharge at oil and gas wells and urban stormwater runoff reach freshwater ecosystems by design. We have found the toxic effects of fuels in freshwater environments is difficult to predict using existing laboratory methods and results. Crude oil and refined products like diesel fuel are a mixture of numerous classes of chemicals including polycyclic aromatic hydrocarbons (PAHs), naphthenes/cycloalkanes, BTEX (benzene, toluene, ethylbenzene, xylene) and metals (United States Environmental Protection Agency 2016; International Programme on Chemical Safety (IPCS) 1995). Each chemical has a different fate (retention and loss) and bioavailability in aquatic ecosystems or experimental aquaculture tanks. During spill events, fuel mixtures are often simplified to total petroleum hydrocarbons (TPH, mg/L) of diesel range organics (DRO) based on carbon chain lengths (C10–C28), while laboratory experiments used to prescribe environmental risk are typically conducted using a single petrochemical component at a time.

Single component studies offer more control and repeatability but fail to incorporate possible synergisms of fuel components.

Until recently, acute toxicity trials that inform risk assessment and water quality standards including those for diesel almost exclusively considered mortality after 96 hr of exposure (Stephen et al. 1985; Adams et al. 2017). Yet sublethal exposures to numerous toxicants impair physiology and behaviors needed for reproduction, social interactions, predator–prey dynamics, and other functions that impair fitness (Scott and Sloman 2004; Floyd et al. 2008; Sandahl et al. 2007; Weis et al. 2011; Landrum et al. 2012; Simonato et al. 2008). Additionally, the standard 96-hour exposure model does not adequately represent exposures that may occur in less than 24 hours, such as a fast moving lotic (stream or river) habitat. Environmental risk assessment of petroleum spills should be informed by research that includes probable chemical mixtures of appropriate durations, and should make an effort to measure any sensitive toxic effect (endpoint) that could be important to the individual, population, community, and ecosystem.

Small-bodied freshwater stream fishes are of growing conservation concern, with global declines driven by invasive species, habitat loss, and pollution (Reid et al. 2019; Closs et al. 2016; Vardakas et al. 2025). The ecological impacts of these declines extend beyond aquatic species because many non-aquatic species depend on healthy aquatic ecosystems, especially in arid regions (Baxter et al. 2005; Krueper 1993). As both primary and secondary consumers, small-bodied freshwater fish transfer basal energy from periphyton, detritus, and aquatic invertebrates to higher trophic levels. Native stream fishes serve as key indicators of the environmental health of their habitats, and offer insights into changes in water quality, habitat integrity, and broader ecosystem functions (López-López and Sedeño-Diaz 2015).

Nationally, the United States sees approximately 70 petroleum spills daily (Unites States Environmental Protection Agency 1999). The headwater state of Colorado is an intersection of population growth, busy roadways, pipelines, oil and gas infrastructure, and threatened or endangered fish species. From 2004 to 2024, the state's crude oil production increased by over 755%, rising from 22,534 to 170,289 thousand barrels, accounting for 3.6% of total U.S. production in 2024 (United States Energy Information Administration 2025). Oil and gas spills increased from 380 in 2021 to 1,720 in 2024, an increase of over 352% in just three years (Colorado Energy and Carbon Management Commission 2025) however, improved reporting may explain some of this increase.

In recent decades most developed nations have modernized water quality standards for the chemicals common in roadway fuel spills, coalbed methane produced waters, and oil and gas extraction. Colorado will likely see the need for modernization and expansion of toxicological data in the near future. In preparation for this demand, we have conducted a suite of research and development partly to build tools needed to measure toxic effects of petrochemicals. To simulate the brief pulse of a diesel fuel spill in a small lotic system, small bodied adult fish were exposed to a loading rate of 75 mg/L (14.64 mg/L TPH observed) diesel fuel for 24 hrs. This is well below the 96 hr exposure considered in acute experiments but is likely in Colorado (Duggan et al. 2017). Predator avoidance was assessed to determine if sublethal levels of petroleum can manifest into mortality within the subsequent 72 hrs. The pathogenesis of decreased predator avoidance was examined by lateral line vital dye studies and histopathology.

4.3 Materials and Methods

4.3.1 Test Organisms

Prey species included the Plains Minnow *Hybognathus placitus* (PMW), a species listed as endangered in Colorado; the Flathead Chub *Platygobio gracilis* (FHC), a Colorado species of concern; and the Fathead Minnow *Pimephales promelas*(FHM), a tolerant species widely distributed across North America (Ankley and Villeneuve 2006; Treble et al. 2025). PMW (mean total length (MTL) 40 ± 12 mm) were obtained from the Colorado Parks and Wildlife Native Aquatic Species Restoration Facility (Alamosa, CO), FHM (MTL 35 ± 3 mm) were sourced from Aquatic Bio Systems (Fort Collins, CO), and FHC (MTL 44 ± 15 mm) were collected via backpack electrofishing from Fountain Creek in Colorado Springs, CO. Predator species included native Green Sunfish *Lepomis cyanellus* (GSF; MTL 160 ± 38 mm) collected by trap net from Running Deer Open Space (Fort Collins, CO), introduced Yellow Perch *Perca flavescens* (YEP; MTL 200 ± 20 mm) captured by hook and line from Seaman Reservoir (Larimer County, CO), and native Creek Chub *Semotilus atromaculatus* (CRC; MTL 170 ± 25 mm) obtained by backpack electrofishing from the South Platte River near Sterling, CO. Predator-prey combinations were selected to represent realistic predator and prey co-occurrence observed in eastern Colorado based on 30 years of state biomonitoring records (Table 4.1; Figure 4.1). These predator prey combinations co-occur with oil and gas extractions, legacy coalbed methane, and highways that are proximate to surface waters.

Table 4.1 Colorado Parks and Wildlife sampling records for predator and prey species. GSF co-occurs with PMW across the Arkansas River, Republican River and the South Platte River drainages as recorded by 31 sampling events within three different locations over the past 71 years (internal data, Colorado Parks and Wildlife). Routine state biomonitoring has observed FHM co-occurring with GSF, YEP, and CRC in lotic and lentic systems including the Arkansas, Republican, and South Platte drainages in the past 120 years. The FHC co-occurs in nature with GSF within the Arkansas River drainage.

Prey	Predator	Years of Co-occurrence Observed	Total Number of Co-occurrence Bio-monitoring Events
Plains Minnow (PMW, *Hybognathus placitus*)	Green Sunfish (GSF, *Lepomis cyanellus*)	1950-2023	31
Fathead Minnow (FHM, *Pimephales promelas*)	Yellow Perch (YEP, *Perca flavescens*)	1912-2023	503
Fathead Minnow (FHM, *Pimephales promelas*)	Creek Chub (CRC, *Semotilus atromaculatus*)	876-2023	3117
Fathead Minnow (FHM, *Pimephales promelas*)	Green Sunfish (GSF, *Lepomis cyanellus*)	1876-2023	3117
Flathead Chub (FHC, *Platygobio gracilis*)	Green Sunfish (GSF, *Lepomis cyanellus*)	1889-2023	197
Fathead Minnow (FHM, *Pimephales promelas*)	Green Sunfish (GSF, *Lepomis cyanellus*)	1903-2023	3117

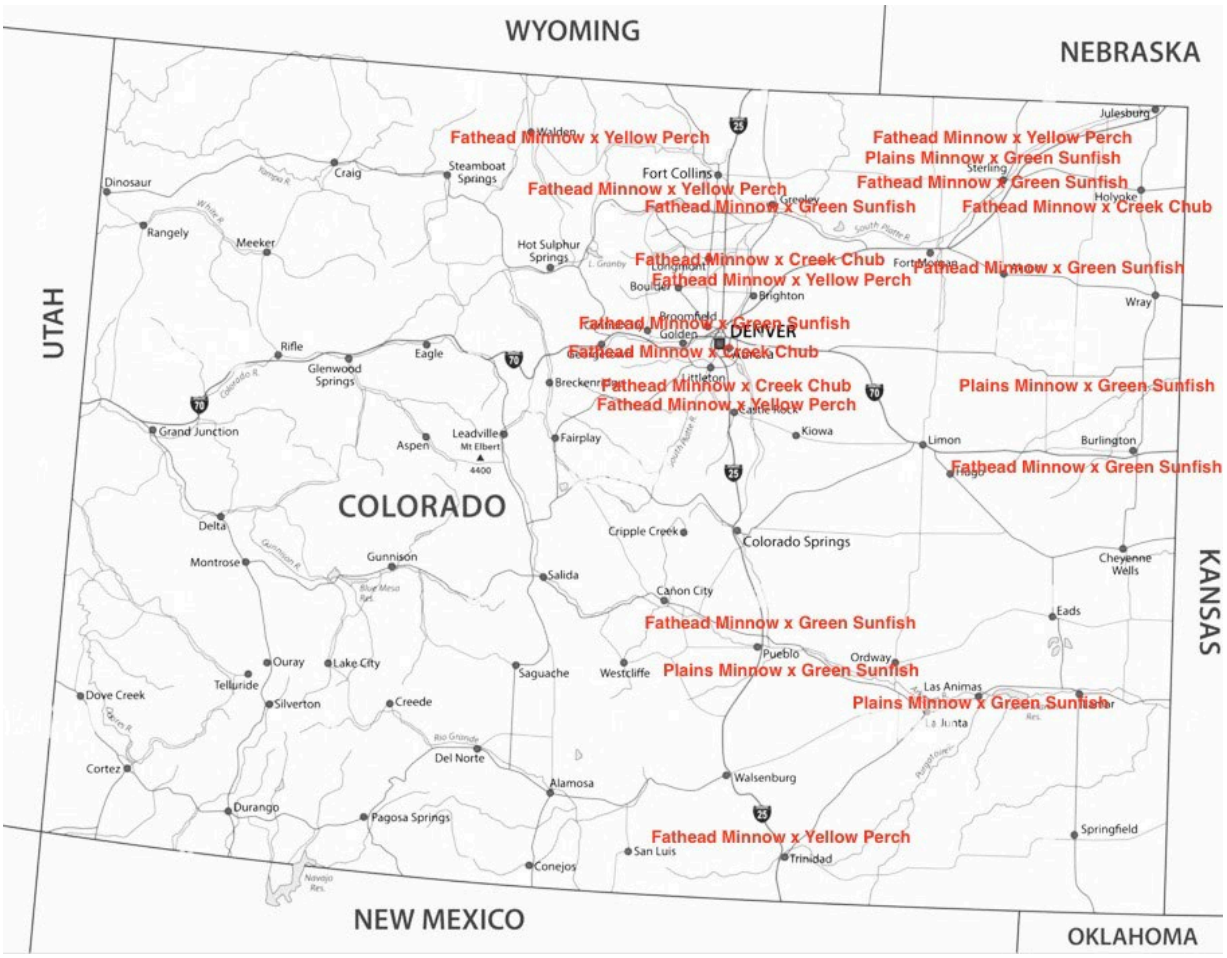

Figure 4.1 Map of observed co-occurrence of predator and prey species used in this study.

4.3.2 Diesel Exposures

All prey fish were marked with ultraviolet fluorescing blue, red, orange or green Visual Implant Elastomer (VIE, Northwest Marine Technology, Ancotes, WA, USA) subdermally injected along the dorsal fin, and allowed to recover for ≥ 14 d. Fish were randomly assigned to eight 6 L stainless steel aquaria (Polarware, Sheboygan, WI, USA, Model G12066) which were filled with 3000 mL of dechlorinated municipal tap water (Fort Collins, CO) mixed with the appropriate aliquot of diesel fuel (Grade No. 2-D, ASTM D975) for target levels of 0 mg/L (control, 4 replicates) or 75 mg/L (exposure, 4 replicates) total compound of diesel fuel oil. This nominal concentration was found to be sub-lethal for salmonids after 96 hour exposure in the same experimental system (Duggan et. al. 2017). All aquaria were held in a temperature controlled water bath (17.26 +/- 1.1 °C). Sodium silicate Pasteur pipettes attached to a blower delivered aeration. Diesel fuel is potentially more bioavailable when photoexcited by the UVA+B spectra (UVA:320-400nm UVB:290-320nm) common in natural sunlight (Krylov et al. 1997). Wide spectrum light bulbs (ZooMed Laboratories, 48” Reptisun 10.0 UVB, F32T8/REP, San Luis

Obispo, CA, USA) with UVA+UVB were hung 15 cm over the aquaria to simulate natural outdoor light at a 16:8 h light: dark photoperiod. These provided ultraviolet light (UV-A+B) intensity of 1.5 mW/cm² at the surface of the water (as measured by UVA/B Meter Model:850009, Sper Scientific, Scottsdale, AZ, USA), which is relatively one tenth of what would be observed with direct sunlight (14.5 mW/cm²). After the 24 hour exposure, prey fish were placed in identical aquaria (6 L hotel pans) with fresh, uncontaminated water for at least fifteen minutes before assessing predator avoidance.

4.3.3 Diesel Concentrations and Water Quality

Water samples from every replicate tank were taken from the middle of the water column for assessment of TPH by gas chromatograph. Samples were sent to a state certified analytical chemistry laboratory (ACZ Laboratories, Inc., Steamboat Springs, CO, USA) for analysis of extractable diesel fuel range organics (DRO) total petroleum hydrocarbons C10-C28 (TPH) using the Environmental Protection Agency's M8015D/API-DRO GC/FID method. Water chemistry data (temperature, pH, dissolved oxygen, hardness and alkalinity) was collected from all exposure and control tanks across all experiments. No trace diesel was observed in controls or in predator avoidance test tanks (MDL of 0.249 to 0.25 mg/L). Samples from the 24-hr exposure tanks showed observed levels of TPH that averaged at 14.64 +/- 7.65 mg/L in the water column (Table 4.2). It is common to lose a majority of diesel fuel from the water column due to volatilization, coalescence, and partitioning to the water surface and container walls during toxicity trials (Hodson et al. 2019; Duggan et al. 2017). Dissolved oxygen, temperature, alkalinity, hardness, and pH remained consistent and levels ideal for fish culture across all exposure and control aquaria.

Table 4.2 Water Chemistry. Total Petroleum Hydrocarbons (TPH) of extractable Diesel Range Organics (DRO), temperature, dissolved oxygen, hardness, alkalinity, and pH observed across all experiments. ND = Not Detected (below MDL of 0.235 mg/L).

Parameter	**Control (mean +/- SD)**	**Exposed (mean +/- SD)**
Diesel Loading Rate mg/L	0	75
Average Observed Diesel Concentration (TPH C10 to C28 mg/L), n=33	ND	14.64 +/- 7.65m
Average Temperature (Celsius), n=32	17.19 +/- 1.07	17.21 +/- 1.15
Average D.O. mg/L, n=9	9.34 +/- 0.16	9.21 +/- 0.16
Average Hardness mg/L $CaCO_3$, n=17	55.41 +/- 4.38	55.72 +/- 3.52
Average Alkalinity mg/L $CaCO_3$, n=17	41.11 +/- 6.72	40.59 +/-5.15
Average pH, n=9	7.69 +/- 0.08	7.64 +/- 0.11

4.3.4 Predator Avoidance Tests

Predator-prey combinations including number of trials for each are listed in Table 4.1. These combinations were selected to represent realistic predator-prey interactions in eastern Colorado based on 30 years of state biomonitoring records. Predator species were not exposed to toxicants and were fasted for 24 hours prior to trials. Diesel-exposed and control prey of similar mean total length (MTL; <0.45 mm mean difference) were paired with a large-bodied predator. For each combination prey fish MTL were 23.5% (0.055 SD) of the predator MTL. This is well below the maximum prey size recommended of 33% (Ruzycki et al. 2003). All VIE tag colors were evenly distributed across trials to ensure that no single color was disproportionately associated with exposed or control fish.

Initial trials consisted of one randomly selected diesel-exposed prey, one randomly selected control prey, and one predator placed into a circular black plastic tank (204 L; KMB 101, Tuff Stuff Products, Terra Bella, CA, USA; Supplemental Figure 4.2) filled to 33% of tank depth. For the first 15–30 minutes, the prey fish were confined within a mesh cylinder that protected them from the predator. After the acclimation period, the mesh barrier was removed, allowing all fish to interact freely. Each trial continued until one of the two prey fish was consumed. The surviving prey fish was then removed from the predatory chamber to verify treatment based on tag color. Following predator evasion trials all fish were euthanized using buffered tricaine methanesulfonate (MS-222; AVMA 2020).

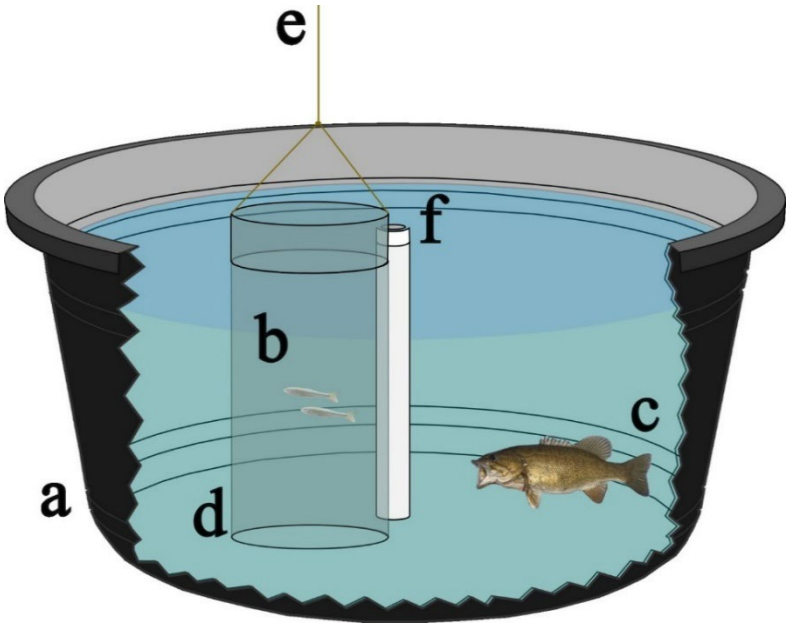

Figure 4.2 Predator-prey interaction chamber. Shown here with a side cutaway from the 204 L tank (a. KMB 101, Tuff Stuff Products, Terra Bella, CA, USA). Prey fish (b) were protected from predator fish (c) within a mesh cylinder (d. 1 mm pore size) for the first 15-30 minutes. The protective mesh cylinder was then lifted by a string (e). Between trials the stand pipe (f) was removed, tank was thoroughly rinsed to remove scents and the tank was refilled to 30% of height of the tank with dechlorinated municipal tap-water at the same temperature as holding and rearing aquaria.

Abnormal shoaling behavior was incidentally noted among diesel-exposed fish. In two trials, groups of four control and four exposed FHC were introduced alongside either two or four Green Sunfish (NSF) and in six additional trials, group compositions included either four exposed and four control prey with four predators, or three exposed and three control prey with three predators. Trials were terminated when 50% of the total prey were consumed or when 3 hours had elapsed at which time remaining fish were removed to verify identification based on tag color followed by measuring length (mm) and weight (grams) before euthanization with buffered MS-222.

Statistical analysis of predator-prey trial data was conducted using a generalized linear model (GLM) with a binomial error distribution and logit link function in RStudio v1.1.463. A logistic regression was used to test the *a priori* directional hypothesis that exposure to diesel would increase the probability of mortality in fish relative to control fish. Treatment (diesel-exposed or control) was included as a fixed effect, with the binary outcome (1 = consumed, 0 = survived) as the response variable. Model estimates are reported as log-odds with standard errors, z-values, and one-tailed p-values. Odds ratios were calculated by exponentiating the model coefficients.

4.3.5 Vital Dye Staining

Vital dye analysis has been used to assess morphology of the lateral line and the effect of numerous toxicants (Mekdara and VanTrump, 2022; Hernandez et al. 2006). FHM (n = 16) were randomly distributed across aquaria for 24 hour control or diesel exposure as previously described. After 24 hours of exposure, fish from each replicate (n=8) were placed in 0.0024% 4-Di-2-Asp (4-(4-Diethylaminostyryl)-N-methylpyridinium iodide, VWR International, 102989-616) solution for five minutes. Separate dye containers were used for control fish and diesel fish to avoid diesel contamination. Fish were rinsed in fresh system water then euthanized via immersion in pH buffered MS-222. Fluorescence of hair cells in superficial and canal neuromasts was evaluated with a MEIJI EMZ-TR dissecting scope on a boom stand. The scope was adapted with a royal blue-green only filter and a royal blue light was used to illuminate target areas (SFA-RB-GO, NightSea, Inc., Hatfield, PA, USA). Images and video of both sides of each fish were captured with an Unitron Excelis HD color digital camera and CaptaVision+ software (Accu-scope, Inc, Commack, NY, USA). Serial images of fish were stitched together using AutoStitch to produce images of the whole left and right sides (Brown and Lowe2007). All euthanized fish were measured (standard length) and weighed. Total neuromast counts from each fish (left + right sides) were assessed by Cell Profiler (V3) with manual quality control. A subset of each body was reassessed (left + right) to capture just the caudal fin rays (caudal to the peduncle) and cranial region (snout to insertion of the pectoral fin) as these regions were predicted to respond differently. Diesel-exposed and control fish neuromast counts (total body, total tail, and total head) were compared with a Wilcoxon rank-sum test due to the small sample size and potential non-normal distribution of the data. As there were no significant differences in length or weight between diesel-exposed and control fish, these variables were not included as covariates in the statistical analyses (Table 4.3).

Table 4.3 Weight and length of FHM used for vital dye and histopathology. There was no statistical difference in total length of fish (Mann-Whitney Test for Two Independent Samples. Control: Count=8 Rank Sum=83 U=17 Exposed: Count=8 Rank Sum=53 U=47. p =0.130 with exact variance) or fish mass (Mann-Whitney Test for Two Independent Samples. Control: Count=8 Rank Sum=81 U=19 Exposed: Count=8 Rank Sum=55 U=45. p =0.195 with exact variance).

	Control FHM	**Diesel exposed FHM**
Average total length (mm, SD)	**37.125 (+/- 3.136)**	**33.375 (+/- 5.655)**
Average weight (g, SD)	**0.55 g (+/- 0.146)**	**0.4475 (+/- 0.164)**

4.3.6 Histopathology

FHM from the vital-dye studies (n= 8 controls and n=8 exposed) were immersed whole in 10% neutral buffered formalin for >48 hours. Fish were trimmed coronally (i.e., through the transverse plane) through the middle of the eyes, branchial chamber, and the body to represent viscera (including heart, liver, intestine, kidney, spleen, body wall). Fish were scored for the presence or absence of acute keratitis, anterior uveitis, and gill necrosis. These were compared with Fisher's exact tests. The degree of interlamellar filling was scored as absent, mild (less than ⅓ of the interlamellar space occluded), moderate (⅓ to ⅔ occluded), or severe (>⅔ occluded). The degree of interlamellar filling was compared by Wilcoxon rank-sum test. The presence or absence of rare monogenean branchial parasites was noted and compared by Fisher's exact test. The presence or absence of apoptosis in superficial neuromasts was recorded and compared by Fisher's exact test. Only fish with 2 or more superficial neuromasts histologically represented were included in the evaluation.

4.4 Results

4.4.1 Diesel exposure impairs predator avoidance

Prey fish, including Plains Minnow (*Hybognathus placitus*, PMW), Fathead Minnow (*Pimephales promelas*, FHM), and Flathead Chub (*Platygobio gracilis*, FHC), were exposed to diesel at a 75 mg/L loading rate (observed at 14.64 ± 7.65 mg/L in the water column; Table 4.1) for 24 hours. No mortality was observed in any exposures. One diesel exposed fish and one control fish of the same species were then placed in trials with individual predator species Green Sunfish (*Lepomis cyanellus*, GSF), Yellow Perch (*Perca flavescens*, YEP), and Creek Chub (*Semotilus atromaculatus*, CRC). Predators consistently consumed diesel-exposed fish before controls (Table 4.4; Figure 4.3), and this preference was statistically significant in four of six

predator–prey scenarios. GSF consumed diesel-exposed PMW in 95% of trials ($p < 0.001$), YEP consumed exposed FHM in 82% of trials ($p = 0.00325$), and NSF consumed 95% of exposed PMW ($p = 0.00002$).

Table 4.4 Results of prey predator pairings following 24 hour sub-acute diesel exposure. Asterisks (*) denote statistically significant ($p < 0.05$) differences between control and diesel exposed.

Prey Fish x Predator Fish	Number of Trials	Total Number of Prey Fish (Equal number of diesel-exposed and control)	Estimate (log-odds)	Standard Error	z value	One Tailed p value	Odds Ratio
Plains Minnow x Green Sunfish	20	40	5.89	1.451	4.059	0.00002466*	361
Fathead Minnow x Yellow Perch	11	22	3.01	1.10	2.721	0.00325*	20.2
Fathead Minnow x Creek Chub	14	28	0.58	0.7638	0.753	0.226	1.8
Fathead Minnow x Green Sunfish	23	46	0.8837	0.6042	1.462	0.0718	2.4
Flathead Chub (4 x 4) x Green Sunfish (4)	2	16	3.162	1.411	-2.242	0.0118*	3.04
Fathead Minnow (4x4 & 3x3) x Green Sunfish (4 & 3)	6	44	4.847	1.405	-3.449	0.00009972*	26.7

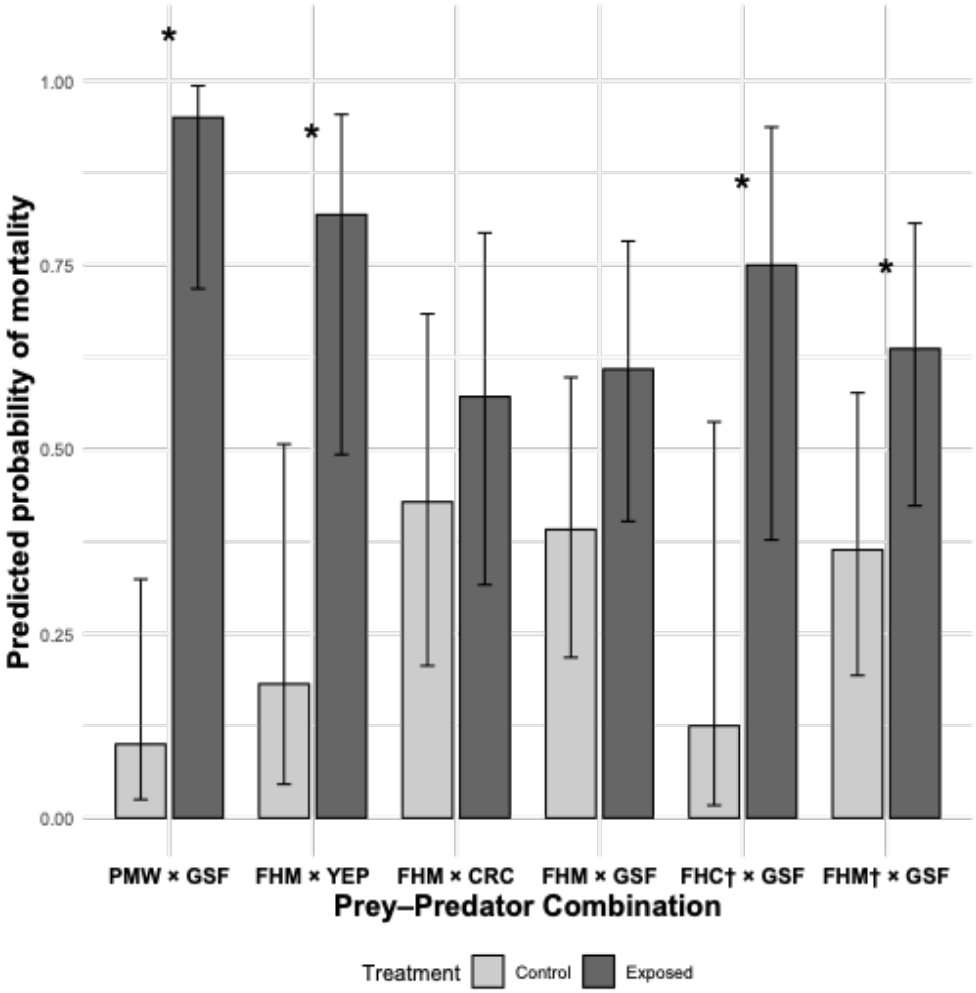

Figure 4.3 Proportion of diesel exposed and control prey fish consumed: Summary of six predator-prey combinations testing the mortality proportion of diesel-exposed versus control fish when paired with predators. Data represent mean proportion eaten for each group ± standard deviation (error bars). Asterisks indicate statistically significant (p <0.05) differences between groups. Full statistical results are presented in Table 1. PMW = Plains Minnow; GSF = Green Sunfish; FHM = Fathead Minnow; YEP = Yellow Perch; CRC = Creek Chub. Asterisks (*) denote p value < 0.05 (†) denotes shoaling experiment in which multiple exposed and multiple control fish were allowed to school.

Failure to shoal (swim in a coordinated school) was noted in the 24 hour exposure treatment tanks. To determine if shoaling behavior was compromised by diesel, multiple GSF were allowed to prey on mixed groups (50% control FHM and 50% diesel exposed FHM; up to 4 control and 4 diesel exposed). There was 75% of exposed FHC consumed compared to 12.5% of control FHC ($p < 0.001$). Similarly, GSF consumed 72.7% of diesel-exposed FHM compared to 9.1% of controls ($p = 0.000099$). These findings suggest that compromised shoaling behavior following diesel exposure may increase vulnerability to predators and ultimately elevate mortality risk. Although not statistically significant, similar trends were observed in the remaining predator-prey pairings: CRC consumed 64.3% of exposed FHM ($p = 0.226$) and GSF consumed 60.9% of exposed FHM ($p = 0.0718$).

4.4.2 Diesel exposure decreases viable neuromasts

Neuromasts are sensory structures distributed superficially on the skin and in specialized cutaneous canals. They sense changes in local water pressure and flow, such as the bow wave of a predator strike, and allow fish to reflexively respond to movement. Loss of neuromast function could reduce ability to evade predators and impair shoaling behavior. Vital dye uptake is commonly used to assess the function of neuromast hair cells and is a reliable proxy for mechanotransduction and hair cell integrity (Hernández et al. 2006). Reduced uptake indicates hair cell degeneration, dysfunction, or necrosis and is a useful indicator of sensory impairment (Mekdara and VanTrump 2022; Hernandez et al. 2006; Young et al. 2018). FHM exposed to diesel for 24 hours had markedly diminished numbers of neuromasts detected by vital dye uptake compared to FHM control (Figure 4.4 and 4.5). The average count of total neuromasts on diesel exposed fish (580.25 +/- 483.24) was significantly less than the average total count on control fish (1157.75 +/- 290.25; p =0.007382). This was especially true for the tail region of fish where diesel-exposed FHM neuromasts (200.88 +/- 49.2) was significantly less than that of control fish (489.13 +/- 103.803; p =0.0006741; Figure 4.6) Diesel-exposed fish also had significantly less cranial neuromasts (285.13 +/- 190.24) than control fish (489.13+/- 103.803; p =0.03248). Lateral line impacts would go unnoticed in laboratory toxicity experiments that assessed mortality alone. But in nature predator avoidance, shoaling, and other behaviors reliant on this sensory system would be impaired.

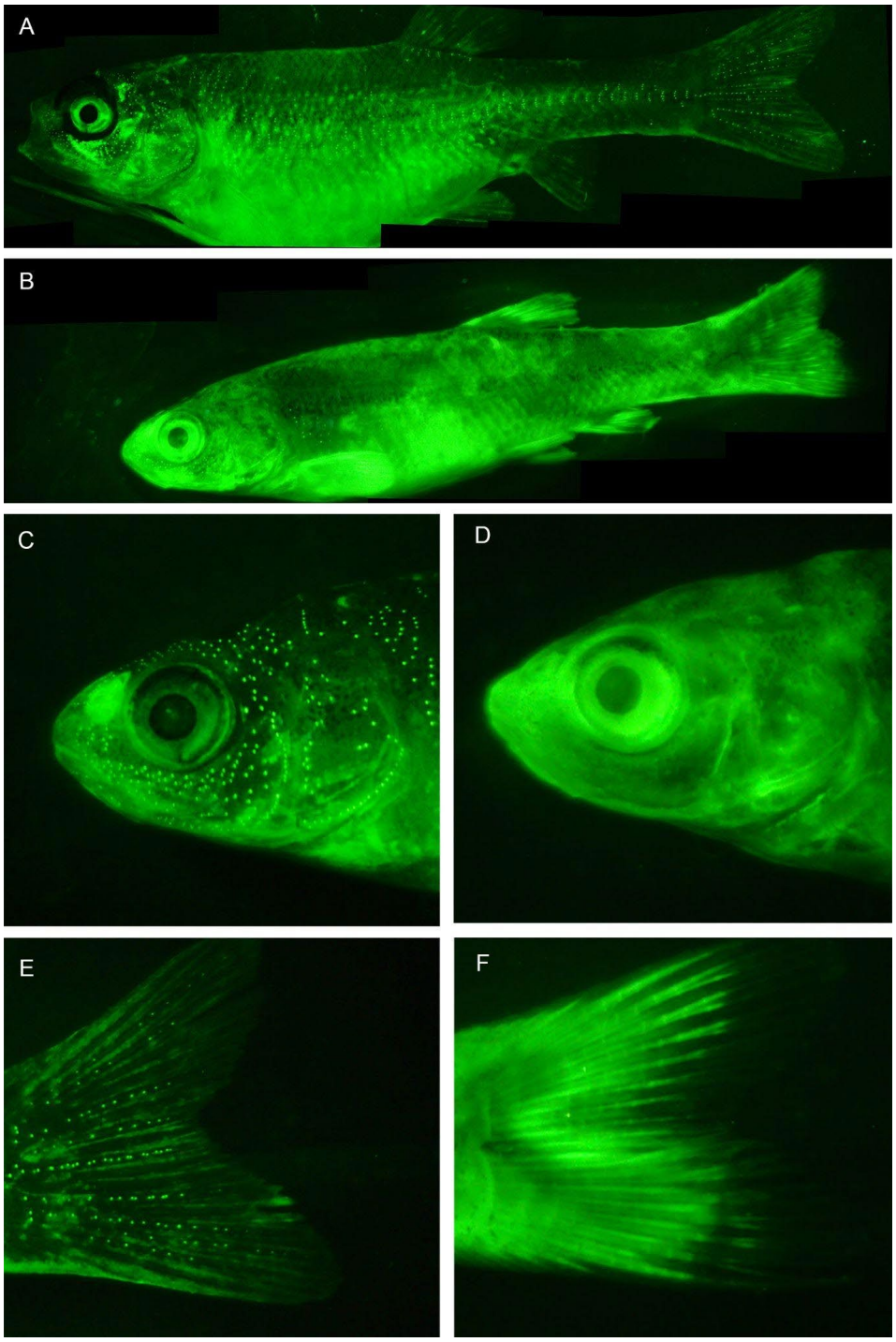

Figure 4.4 Distribution of neuromasts in a control Fathead Minnow (A, C, E), and Fathead Minnow exposed to diesel for 24 hrs (B, D, and F). Note in figure 3D the cornea is opacified. 4-Di-2-ASP vital dye immersion and visualization with blue light.

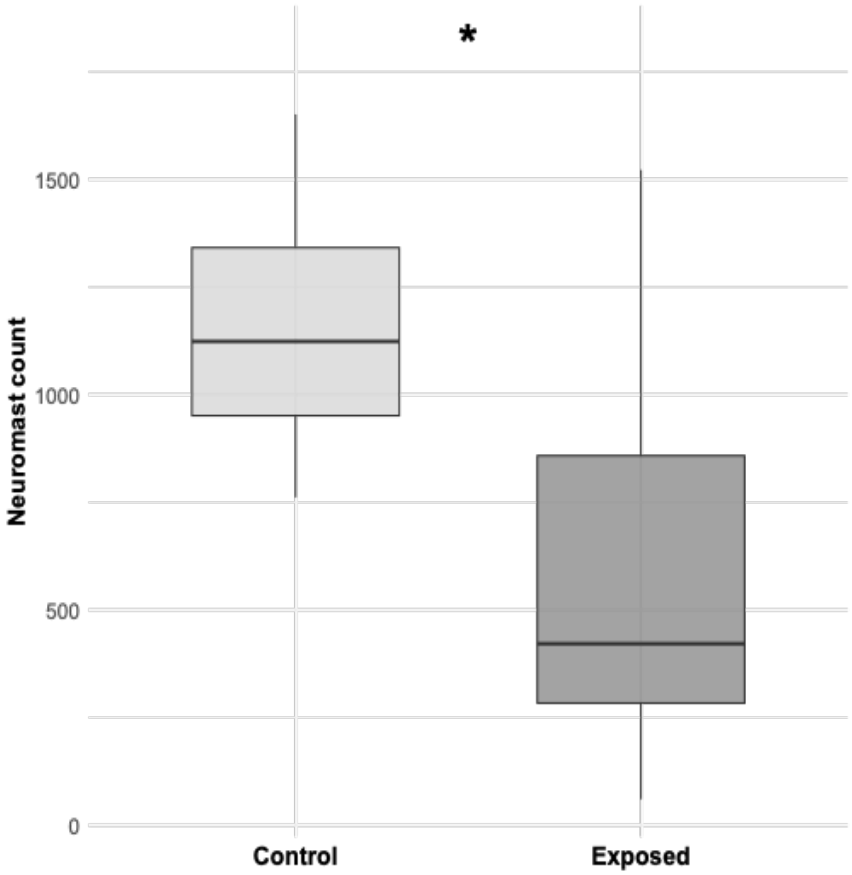

Figure 4.5 Lateral line neuromast cell counts. Average neuromast cell counts on control and diesel-exposed Fathead Minnow *Pimephales promelas*. Asterisks indicate statistically significant (p <0.05) differences between groups.

4.4.3 Diesel exposure causes ocular and gill pathology

Pathologies effecting sight, oxygen transfer, and the mechanosensory system were investigated as a potential explanation for reduced predator avoidance. Corneal opacity was noted in diesel exposed fish, and hypothesized that this reflected corneal edema or keratitis. Figures 4.6 and 4.7 summarize key histopathologic findings. Subgross histopathology of a representative FHM eye is presented in Figure 4.7a for orientation. Control fish had a clear, unobstructed iridocorneal angle (Figure 4.7b). The corneal epithelium of control fish was 4-5 cell layers thick and comprised of uniform cuboidal to polygonal cells with discrete cell borders, modest amounts of eosinophilic cytoplasm, and round to oval nuclei with homogenous chromatin, and the corneal stroma was comprised of uniform collagen (Figure 4.7c). Control gill had normal intact secondary lamellar architecture (Figure 4.7d).

All eight exposed fish had anterior uveitis while no control fish had anterior uveitis (Figure 4.7C and 4.6, p = 0.0011). In exposed fish there were clusters of lymphocytes and macrophages in the anterior chamber between the cornea and iris or adhered to either the surface of the caudal cornea or anterior iris (Figure 4.7e). All exposed fish had marked dilation of iridal vasculature

(physiologic hyperemia). All eight FHM exposed to diesel had acute necrotizing keratitis while no keratitis was identified in control fish (Figure 4.7e and 4.6; $p < 0.0001$). The corneal epithelium of exposed fish was attenuated to 2-3 recognizable cell layers and there were numerous individual shrunken epithelial cells with hypereosinophilic cytoplasm and was broadly stippled by basophilic karyorrhectic and pyknotic debris. In exposed fish the corneal stroma was infiltrated by low numbers of leukocytes (Figure 4.7d). One of the eight exposed fish had diffuse necrosis and complete loss of the epithelium and necrosis of the corneal stroma, which was homogeneously eosinophilic without distinction of collagen fibers. Both keratitis and anterior uveitis would be expected to cloud vision and thus could contribute to poor predator avoidance. These findings would not be captured in a study focused solely on mortality.

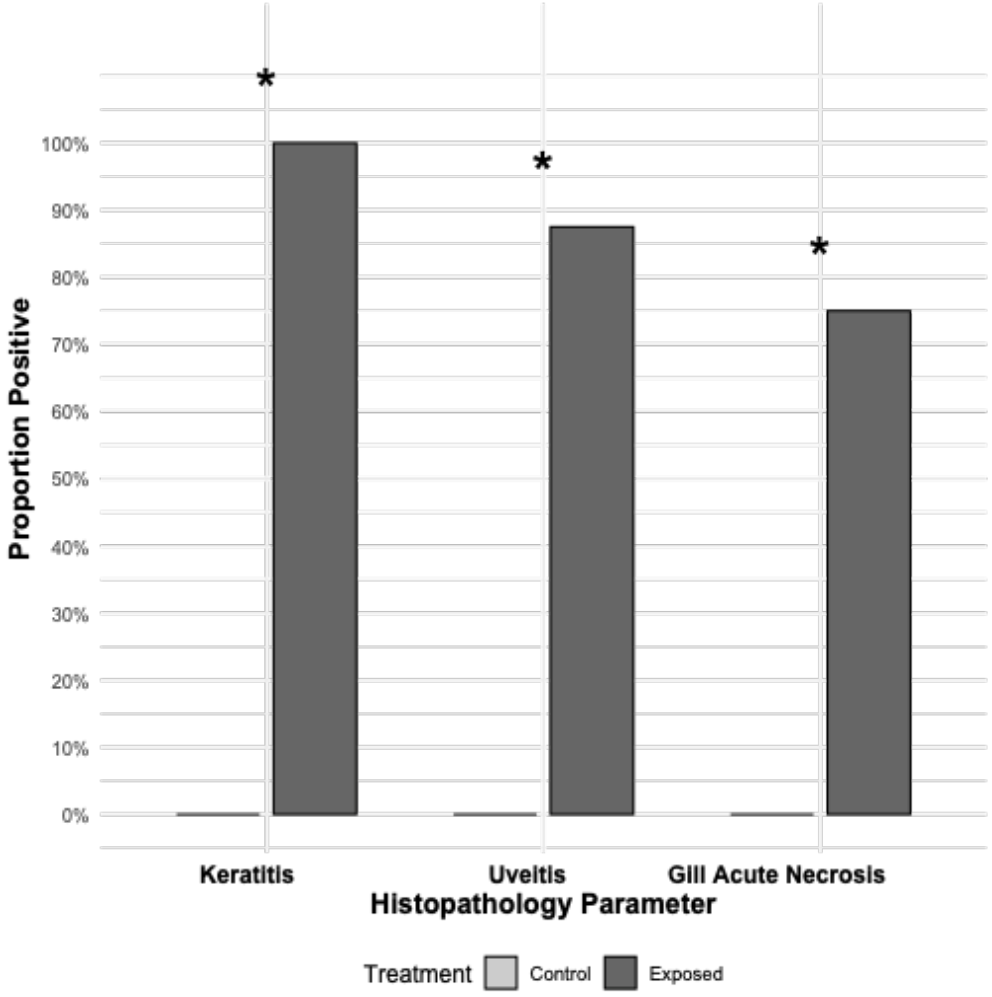

Figure 4.6 Ocular and Gill Pathologies. Proportion of control (light grey) and diesel exposed (dark grey) Fat Head Minnows exhibiting keratitis, uveitis and acute necrosis of the gill. Asterisk (*) denotes significant difference between the control and treatment groups for each pathology.

Seven of eight treated fish had focal or multifocal mild acute necrosis of epithelial cells lining secondary lamellae (Figure 4.7g), which was not identified in any control fish ($p = 0.004$). Two of eight exposed fish had mild focal telangiectatic change, which was not identified in any control fish, but this was not statistically significant ($p = 0.47712$). Both diesel-exposed and control fish had the same degree of interlamellar filling by proliferative epithelial cells (mild to moderate proliferative bronchitis; $p = 0.408$, considered a background lesion). Rare gill-associated monogenean parasite profiles were identified equivalently in both groups (50% of control, 40% of exposed; $p = 1.0$). Acute branchitis is also a finding that would not be identified in a standard toxicology test.

Two of seven control FHM with greater than two superficial neuromasts (mean=5.3 per fish) exhibited apoptosis within superficial neuromasts while five of six exposed FHM with greater than two neuromasts in histologic section had this change (mean=7.3 per fish; $p = 0.103$). Canal neuromasts were encountered so infrequently in histologic section that these were not sufficient for evaluation. No other significant lesions were identified in any other tissues.

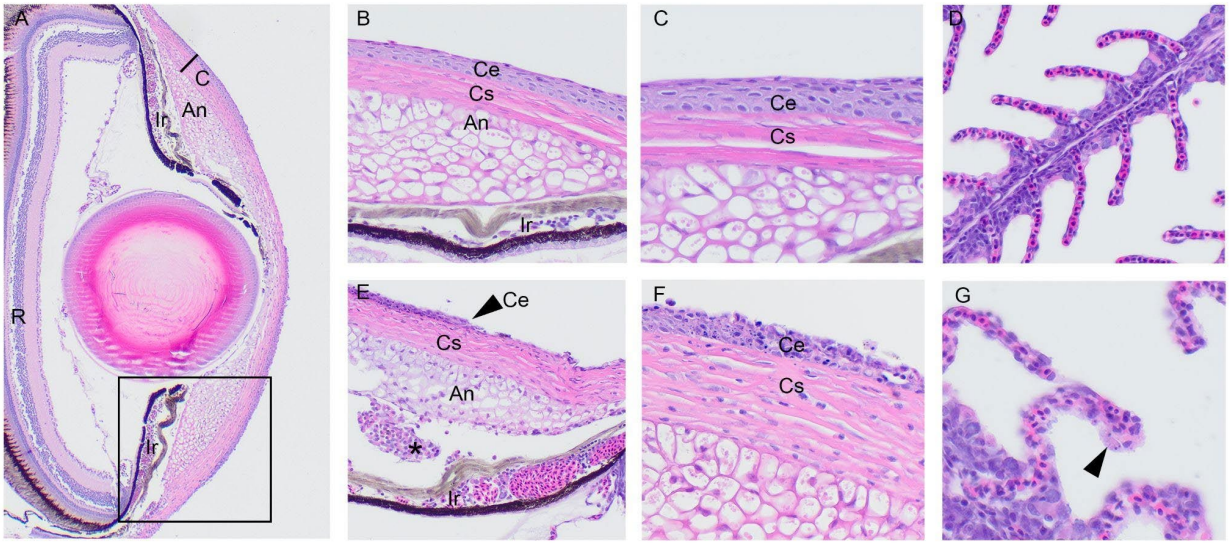

Figure 4.7 Histopathology of control and diesel-exposed Fathead Minnow. A. Anatomy, control FHM eye. The corneal surface is directed towards the right. C, cornea, An, annular ligament, Ir, iris, L, lens, R, retina. Box indicates the iridocorneal angle. B. Higher magnification of iridocorneal angle as pictured in A but rotated 90 degrees counterclockwise; corneal surface is now directed upwards. Control FHM. The cornea is comprised of the corneal epithelium, Ce, and the corneal stroma, Cs. C. Superficial cornea, control FHM. The corneal epithelium is 4-5 cell layers thick and uniform. D. Gill, control FHM. Secondary lamellae are uniform in thickness and length. E. Iridocorneal angle, diesel-exposed FHM. The corneal epithelium is attenuated (arrowhead), the corneal stroma is infiltrated by leukocytes, and there are inflammatory cells in the anterior chamber (*, between An and Ir). F. Superficial cornea, diesel exposed FHM. The corneal epithelium is stippled by pyknotic and karyorrhectic nuclear debris. The collagen of the stroma is smudgy with small amounts of nuclear debris and is hypercellular due to infiltrating leukocytes. G. Gill, exposed FHM. Secondary lamellae with acute epithelial cell necrosis. The necrotic cell has hypereosinophilic cytoplasm and pyknotic nucleus (arrowhead).

4.5 Discussion

Prey fish exposed to sublethal diesel were three to 360 times more likely to be predated upon than non-exposed prey fish. Even when defensive shoaling in a school of fish was allowed diesel-exposed prey were eaten first. FHM exposed to sublethal diesel had significantly decreased functional neuromasts and significantly increased prevalence of acute severe keratitis and anterior uveitis. Successful predator avoidance and shoaling activity depends on the lateral line and vision. Diesel-induced damage to the neuromasts and eyes (keratitis and uveitis) was only seen in diesel-exposed fish. It is highly probable that this caused reduced evasion of predators measured in this study. In our study the decreased neuromasts response was more significant on the tail of exposed fish than the cranial region (Figure 4.8). It is possible that this portion of the sensory apparatus may be more important for evasion during a strike or chase than previously assumed. Mild acute gill necrosis may be due to oxidative injury or direct membrane effects, but is of uncertain significance due to the capacity of this tissue for rapid regeneration (Wolf et al. 2015). Gill necrosis may become more important in longer exposures or at higher concentration.

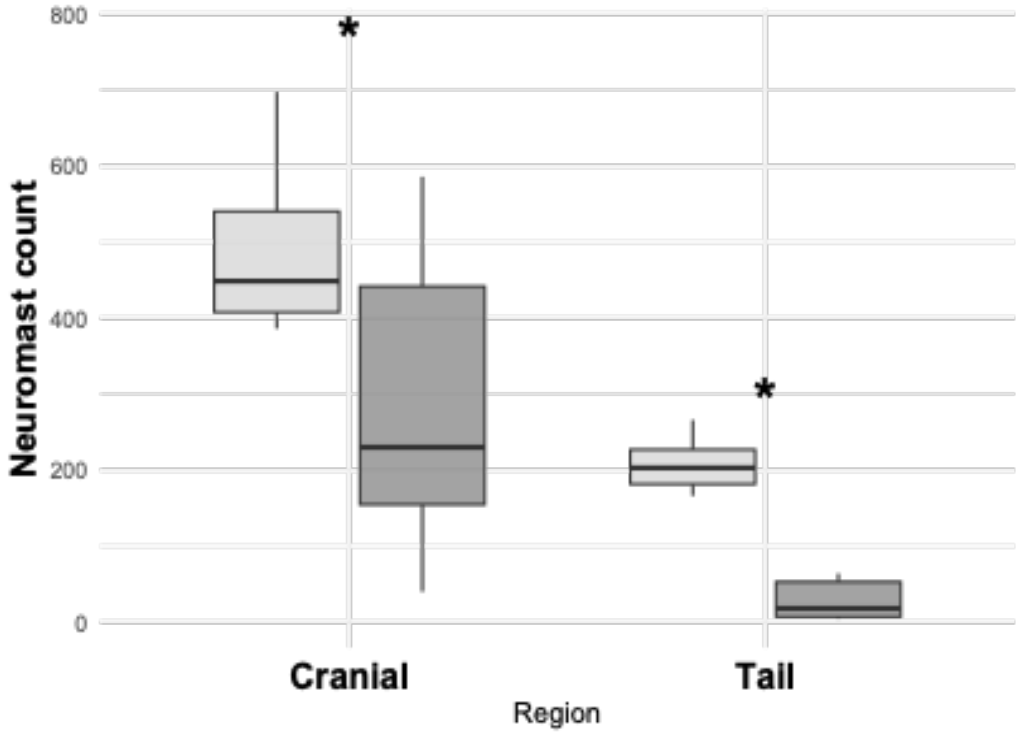

Figure 4.8 Cranial and tail neuromast counts in control (light grey) vs diesel-exposed (dark grey) Fathead Minnow. Each region represents a subsample of neuromasts represented in Figure 4.5. Boxplots show median (line), interquartile range (box), and range/SD (whiskers) of neuromast counts. One-tailed Wilcoxon tests (Exposed < Control) revealed significantly fewer neuromasts in exposed fish in the cranial ($p = 0.012$) and tail ($p = 0.046$) regions. Asterisks (*) denote $p < 0.05$.

Loss of predator avoidance after exposure to diesel was observed across all species tested. We suspect this susceptibility may be conserved across all small bodied fish. The family *Leuciscidae* represents nearly one-third of all freshwater fish species currently at risk of extinction globally from impacts such as habitat destruction and fragmentation, anthropogenic water use, pollution, and the spread of nonnative predators or invasive species (Vardakas et al. 2025). These fish fill critical niches in freshwater ecosystems. Their abundance and trophic position make them key forage species that regulate food-web connectivity and energy pathways across freshwater and riparian ecosystems, linking aquatic production to terrestrial consumers such as birds and mammals (Baxter et al. 2005; López-López and Sedeño-Diaz 2015).

Reported diesel 48 to 96-hour LC_{50} values (concentration that kills 50% of test organisms) for fish range from below 5 mg/L to over 30,000 mg/L (Table 4.5). Discrepant toxicological results for petroleum mixtures stem in part from differences in experimental design and how dose is measured, with some reporting measured chemical concentrations and others only nominal loading rates. Methods also differ in whether they evaluate only the water-soluble fraction, allow hydrocarbons to partition to surfaces or sediment, or forcibly disperse the entire dose into solution. Moreover, the sun's ultraviolet (UV) radiation can photo-excite hydrocarbons in diesel inducing photo enhanced toxicity (Willis et. al. 2014; Hettithanthri et al. 2024), yet this mechanism is often overlooked. Differences in study design, species sensitivities, and fuel mixtures have produced highly variable toxicity estimates. Some acute guidelines for safe levels of total petroleum hydrocarbons (TPHs) exist however they are inconsistent. Most guidelines and standards are created for individual chemicals or chemical classes found in diesel. Systems for predicting synergistic, antagonistic and additive toxicity of petroleum chemicals are lacking. Reconciling a predictive tool with field and laboratory observations should be a priority for future studies. Methods for this study were devised to incorporate mixtures at a short duration. Future petroleum toxicity studies would benefit from incorporating predator avoidance behavior, lateral line function, keratitis and uveitis. Extending this experiment to 96 hr, and 30 d exposures will result in lower, more environmentally sound, toxic thresholds.

Table 4.5 Reported LC_{50} values for representative freshwater fish species including exposure duration, test type, and reference. Data illustrate the wide range of species-specific sensitivities to diesel fuel, from highly sensitive taxa such as salmonids and tilapia to more tolerant species including common carp. Full experimental details and references are provided to enable reproducibility and cross-study comparison. Some results are weathered diesel which may have significantly more potentially toxic hydrocarbons per unit of TPH when measured and reported at the DRO range.

Species	**Concentration mg/L**	**Methods and Notes**	**Source**
Fathead minnow (*Pimephales promelas)*	38.6	Static, flow-through, emulsion, 19°C, pH 7.1	IPCS (1995)
Fathead minnow (*Pimephales promelas)*	2.5	LC_{50} Linear interpolation. Weathered Diesel. LC_{50} >4.33 (>6.28)	Washington State Department of Ecology (2020)
Fathead minnow (*Pimephales promelas)*	2.5 (2.2-2.8)	Spearman-Kärber and Dunnett comparison. Weathered Diesel	Hobbs et al. (2018)
Fathead minnow (*Pimephales promelas)*	2.101 - 2.981	96 hr LC_{50}	Valero Marketing & Supply Company (2014)
Pink Salmon (*Onchorhychus gorbuscha)*	32-123	oil-water mixture; static	IPCS (1995)
Pink Salmon (*Onchorhychus gorbuscha)*	0.95 - 1.62	96 hr LC_{50}	Valero Marketing & Supply Company (2014)
Coho Salmon *(Onchohynchus kisutch)*	2186-3017	Emulsion; flow-through	IPCS (1995)
Rainbow Trout (*Oncorhynchus mykiss)*	3333-33 216	Varies by study: LL_{50}, SDS, EC_{50}	IPCS (1995)
Rainbow Trout (*Oncorhynchus mykiss)*	21	96-hr LC_{50}	Chevron Phillips Chemical Company (2024)
Rainbow Trout (*Oncorhynchus mykiss)*	44.8	14-day EC_{50}	Mos et al. 2008
Rainbow Trout (*Oncorhynchus mykiss)*	2.4	48-hr LL_{50}	CONCAWE 1996
Rainbow Trout (*Oncorhynchus mykiss)*	65	96 hr LL_{50}	Valero Marketing & Supply Company (2014)

Salmo sp. Salmon species	2.52	48-hr LC_{50}	Lockhart et al. (1987)
American shad (*Alosa sapidissima*)	167	48-hr TLM	Lockhart et al. (1987)
Blue Gill (*Lepomis macrochirus)*	2.2	96-hr LC_{50}	HollyFrontier Refining & Marketing LLC (2014)
Murray River Rainbowfish larvae (*Melanotaenia fluviatilis*)	0.0213	96-hr LC_{50}	HollyFrontier Refining & Marketing LLC (2014)
Western mosquitofish (*Gambusia affinis)*	4924	96-hr LC_{50}	Valero Marketing & Supply Company (2014)
Striped Bass (*Roccus saxatilis*)	22.2	Not specified	IPCS (1995)
Golden orfe (*Leuciscus idus melanotus)*	120-160	Static	IPCS (1995)
Common Carp (*Cyrinus carpio)*	49.1	Water Soluble Fraction (WSF) diesel	IPCS (1995)
Common Carp (*Cyrinus carpio)*	59.3	WSF diesel	Hameed and Al-Azawi (2016).
Mud skipper (*Periophthalmus koelreuteri)*	4.588	WSF diesel	Bob-Manuel (2012)

Low sample size may have prevented recognition of significant differences in superficial neuromast histopathology. Future studies should consider ultrastructural evaluation by scanning electron microscopy to examine acute pathology of neuromast ultrastructure. Neuromasts are known to regenerate rapidly following some toxicant exposures, and greater work is needed to assess potential for neuromast regeneration following diesel, specifically. Further studies should evaluate potential for resolution of ophthalmic pathology or the potential for chronic corneal scarring and complications of chronic anterior uveitis such as glaucoma.

Decades of aquatic toxicology studies have focused on mortality, the most obvious response variable. This can upwardly bias species mean acute values. A chief way to improve the realism of toxic thresholds is to incorporate sublethal effects. Our results identify novel toxicologic endpoints that could prove valuable for numerous taxon and chemical mixtures.

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5.1 Introduction

The Bluehead Sucker *Pantosteus discobolus* and Flannelmouth Sucker *Catostomus latipinnis* are native fish of the Colorado River Basin that are currently occupying less than 50% of their historical range (Bezzerides and Bestgen 2002). This decline is the result of various factors, including hybridization with non-native suckers. While this is a persistent challenge, the water quality conditions of spawning habitats may also play a crucial role in limiting the recovery of these native species. The majority of Bluehead and Flannelmouth Suckers utilize perennial and intermittent tributaries for spawning, and the success of these events may be highly dependent on local water quality and thermal conditions.

In many river systems, non-native species and their hybrids have become as or more common than native suckers, and broad-scale removal efforts in large rivers are often cost-prohibitive. However, the smaller size of tributaries allows for targeted, low-cost management strategies, such as the use of weirs and traps, to manage spawning populations. For example, a recent project on Roubideau Creek, a tributary to the Gunnison River, demonstrated the feasibility of manually removing non-native and hybrid suckers to decrease hybridized larval production. This successful strategy highlights the importance of tributary spawning habitats, but also raises critical questions about which tributaries are most vital for long-term population recruitment.

To address this, our study investigates the effects of temperature and water quality on the year-to-year survival and recruitment of native sucker populations. We evaluate the thermal landscape experienced by out-migrating larvae to determine if recruitment bottlenecks are associated with the transition from warm tributary habitats to the colder, dam-manipulated mainstem Gunnison River. Temperature data were collected from loggers deployed throughout the tributaries. Similarly, water samples were collected during the larval rearing period (May-June) and analyzed for a full panel of heavy metals and nutrients. This will allow us to understand if factors associated with upstream agricultural land are limiting larval and juvenile survival.

By combining genetic analyses with environmental data, we will identify the relative recruitment provided by individual tributaries. We plan to use low-cost, next-generation genotyping to genetically assign adult suckers from the mainstem Gunnison River back to their natal tributaries (Steele et al. 2019; Wagner et al. 2013). This will allow for the strategic allocation of management resources to the tributaries that provide the greatest benefit to the overall basin population by not only preventing hybridization, but by also providing optimal water quality and temperature conditions for recruitment. Here, we focus on current results from water quality data and numbers of larval collected from nine tributaries of the Gunnison River.

5.2 Methods

5.2.1 Study Site

Our study was conducted across nine previously identified spawning tributaries of the Gunnison River in Colorado. These sites included Gunnison River #3, Tongue Creek, Roubideau Creek, Kannah Creek, Whitewater Creek, the North Fork of the Gunnison River, Uncompahgre River, Escalante Creek, and East Creek. During the sampling period in 2025, Whitewater Creek and East Creek were found to be dry, and therefore no data loggers, water samples, or larval fish were collected from these two sites.

5.2.2 Water quality collection and analysis

To assess thermal conditions, HOBO TidbiT MX waterproof temperature loggers were deployed at each of the seven active sites in June 2025. These loggers were programmed to record temperature every 4 hours for a minimum of one year. The data will be retrieved and the loggers reprogrammed in April 2026 for continued monitoring.

Water samples were collected at the seven active sites for analysis of metals and nutrients. For metal analysis, both filtered (dissolved) and non-filtered (total) samples were collected at each location. Each sample was split into two 60-mL Nalgene® bottles. Filtered samples were collected using a 30-mm, 0.45 µm polyethersulfone (PES) filter attached to a pre-rinsed 50-mL syringe. All metal samples were preserved with three drops of ultra-pure nitric acid (Ultrex® II) to achieve a pH of less than 2 and shipped on ice to the Colorado Parks and Wildlife (CPW) River Watch and Toxicology Laboratory in Fort Collins, CO. Metal concentrations were analyzed using a ThermoScientific iCAP 6000 Series Inductively Coupled Plasma, Optical Emission Spectrometer (ICP-OES) operated with iTeva software (version 2.8.0.97) and introduced via a CETAC ultrasonic nebulizer (U5000AT+).

Nutrient samples were also collected and included total suspended solids (TSS) and samples for nitrate and phosphorus analysis, which were preserved with sulfuric acid. All metal and nutrient samples were analyzed by the CPW River Watch Laboratory. Nutrient concentrations were determined using a Latchat flow-injection analysis (FIA) system.

In-situ water quality parameters were also measured at each site. Temperature was recorded using a lollipop thermometer, and pH was measured with a YSI pH probe. Alkalinity was determined using a two-step titration method. Phenolphthalein alkalinity was measured by titrating a 50-mL sample with sulfuric acid until a colorless endpoint was reached, with the result calculated as the volume of acid used multiplied by 40 and reported as mg/L of $CaCO_3$. Total alkalinity was then measured by continuing the titration with a Bromocresol Green/Methyl Red (BGMR) indicator until a pink-gray endpoint was reached, with the total volume of acid used multiplied by 20 and reported as mg/L of $CaCO_3$.

5.2.3 Larval Sampling

Larval fish were collected from the sites using a hand net. All collected larvae were preserved in 95% ethanol and sent to the Colorado State University Larval Fish Laboratory for species identification. After identification, the samples will be sent to the Idaho Genetics Laboratory for further analysis and developing of the RADseq.

5.2.4 Statistical Analysis

Linear regression models were used to analyze the relationship between the number of larval fish collected and water quality factors. An analysis of variance (ANOVA) was performed to test the significance of the models. The number of larval fish was regressed against water flow. A separate linear model was used to regress the number of larval fish against total alkalinity.

5.3 Results

A total of 501 larval fish, consisting of Bluehead and Flannelmouth Suckers, were collected across the nine study sites during a single sampling event. The number of fish collected varied considerably among the streams (Table 5.1). Statistical analysis revealed that the number of larval fish collected was significantly related to flow ($F_{1,3} = 53.92$, $p < 0.005$) and had a near-significant relationship with phenolphthalein alkalinity ($F_{1,5}$=6.539, $p = 0.05$).

Site Name	Temperature (°C)	Flow (ft³/sec)	pH	Num. larval fish collected
Escalante Creek	19.2	0.70	8.31	103
Gunnison River #3	15.4	468.0	8.42	53
Kannah Creek	20.0	-	8.49	40
North Fork Gunnison	17.1	692.0	8.41	48
Roubideau Creek	18.2	1.28	8.67	111
Tongue Creek	16.0	-	7.69	72
Uncompahgre River	18.9	374.0	8.56	74

Table 5.1 Number of larval suckers collected by site and associated water quality parameters (temperature, flow, and pH) during a single sampling event in June 2025.

Overall, average water quality conditions across the sampled streams during the study period were consistent with the following average values (Table 5.1 and 5.2): Temperature 17.8 ± 1.7°C, flow 307.2 ± 302.5 cfs, pH 8.36 ± 0.3, phenolphthalein alkalinity 15.4 ± 8.1 mg/L $CaCO_3$, total alkalinity 157.7 ± 63.1 mg/L $CaCO_3$. Total and dissolved metal analysis have not been completed at this time. Nutrient analysis showed the following average values (Table 5.2): ammonia 0.04 ± 0.02 mg/L, chloride 8.5 ± 5.2 mg/L, nitrate/nitrite 0.9 ± 1.0 mg/L, sulfate 290 ± 210.5 mg/L.

Site Name	Phenolphthalein Alkalinity (mg/L $CaCO_3$)	Total Alkalinity (mg/L $CaCO_3$)	Ammonia (mg/L)	Chloride (mg/L)	Nitrate/Nitrite (mg/L)	Sulfate (mg/L)
Escalante Creek	28.0	250.0	0.046	14.67	2.63	107.7
Gunnison River #3	8.0	66.0	0.008	2.50	<MDL[1]	32.8
Kannah Creek	16.0	164.0	0.038	16.04	0.08	592.9
N. Fork Gunnison	12.0	88.0	0.007	2.99	0.02	116.8
Roubideau Creek	20.0	200.0	0.03	7.05	0.95	385.4
Tongue Creek	4.0	164.0	0.055	8.46	0.48	477.0
Uncompahgre River	20.0	172.0	0.063	7.71	1.31	317.5

Table 5.2 Water quality parameters measured at each study site, including ammonia, chloride, nitrate/nitrite, sulfate, total alkalinity, and phenolphthalein alkalinity from a single site measurement in June 2025.

[1]*Nitrate/Nitrite MDL: 0.01 mg/L*

5.4 Discussion

These preliminary findings from a single sampling event suggest a potential relationship between larval fish abundance and key water quality parameters. The significant influence of flow and the near-significant relationship with phenolphthalein alkalinity indicate that water quality conditions within these tributaries may play a role in recruitment success. However, these results represent only a snapshot in time. To determine how water quality influences the spawning and recruitment of native suckers, a multi-year dataset is essential. Continued monitoring over the next few years will allow us to evaluate how year-to-year variation in temperature, flow, and other water quality metrics influences the long-term survival and recruitment of these populations.

5.5 References

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COLLABORATIVE PROJECTS WITH COLORADO STATE UNIVERSITY (CSU)

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

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

6.1 Introduction

Water temperature is a fundamental environmental factor that significantly influences biological processes in aquatic ectotherms, including growth and reproduction (Killen 2014; Hasnain et al. 2018; Volkoff and Rønnestad 2020). When water temperatures exceed a species' optimal thermal range, organisms experience thermal stress. This stress can lead to reduced growth, increased metabolic demands, compromised mobility, and, in severe cases, mortality (Fry 1947; Cech and Moyle 2004; Donaldson et al. 2008; Handeland et al. 2008). Identifying the optimal thermal ranges for aquatic organisms is a critical component of ecological management. This information is valuable for informing habitat restoration efforts, predicting population dynamics, and developing comprehensive risk assessments for vulnerable populations (De Vries et al. 2008; Todd et al. 2008; Dibble et al. 2021; Li et al. 2022). Unfortunately, research into thermal tolerance is often taxonomically biased; relatively few studies have focused on characterizing temperature ranges for non-sportfish species or species of conservation concern (Coutant 1977; Beitinger et al. 2000; EPRI 2011; Jonsson 2023). For example, the Colorado Department of Public Health and Environment (CDPHE) lists at least 25 fish species for which no temperature tolerance data are available, with 19 of those species identified as being species of conservation interest.

The development of protective temperature criteria for fish has historically relied on laboratory assessments of acute and chronic thermal responses (Todd et al. 2008). In Colorado, the Water Quality Control Commission (WQCC) recommends the use of two primary acute temperature tests for developing aquatic life standards: the incipient lethal temperature tests (ILT) and critical thermal tests (CT). Both tests are conducted under controlled laboratory conditions, and each experimental approach aims to define a species' thermal tolerance based on a specific acclimation temperature. It is important to acknowledge that these two standardized approaches evaluate physiological responses from temperature stress differently. In ILT tests, fish are abruptly transferred from their acclimation temperature to a constant, higher or lower test temperature. The ILT is defined as the temperature at which 50% of the test organisms experience mortality (Fry 1947). In CT trials, fish are subjected to a constantly increasing (CTMax) or decreasing (CTMin) water temperature, starting from an acclimation temperature. The endpoint for this test is the loss of equilibrium (LOE), which signifies the temperature at which the fish loses its ability to maintain an upright position (Currie et al. 1998). Both ILT and CT methodologies share a critical pre-trial component: acclimation. Acclimation temperature, as defined by the WQCC (WQCC Policy 06-1), is the temperature within a fish's tolerance zone at which the fish is held prior to experimentation, typically for a period of 14 to 30 days (Becker and Genoway 1979; Bennett and Beitinger 1997). Together, data derived from ILT and CT tests are utilized to estimate a species' optimal temperature range, which informs environmental management decisions and the development of protective temperature criteria (Brungs and Jones 1977; EPA 1986; Todd et al. 2008).

In recent decades, a growing body of research has critically examined whether temperature standards derived from controlled laboratory settings accurately reflect the thermal tolerance of fish in wild populations (Konecki et al. 1995; Rodnick et al. 2004; Wehrly et al. 2007; Schofield et al. 2009; Payne et al. 2016). Two primary limitations hinder the extrapolation of ILT and CT trial results under natural temperature regimes: the reliance on a constant acclimation temperature and the failure to account for natural environmental stressors. The acclimation temperature used in laboratory tests is intended to define the relative thermal history and physiological state at of the test organism, providing a stable baseline for comparison across temperature treatments and species (Bates and Morely 2020). However, temperatures in natural environments are rarely constant and vary significantly on both diel and seasonal scales (Wehrly et al. 2007). Consequently, the constant acclimation temperature maintained for weeks prior to laboratory tests does not accurately reflect the dynamic thermal regimes experienced by wild populations (Lutterschmidt and Hutchison 1997; Mandeville et al. 2019). Furthermore, field-based temperature tests may yield results that differ from laboratory findings because wild-caught fish are simultaneously exposed to environmental fluctuations, including varying temperature, dissolved oxygen (DO), water flow, and diverse water quality parameters (Desforges et al. 2023). To bridge the gap between laboratory control and ecological realism, field-based temperature studies are necessary. These studies should use the same ILT and CT methodologies but use wild-caught fish and water directly sourced from where the fish were collected, thereby capturing natural fluctuations in temperature and water quality. To validate that laboratory-derived ILT and CT results are truly protective of wild fish populations, field-based studies using comparable methodologies are needed to determine if significant differences exist between laboratory and field thermal tolerance estimates.

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 interest in western Colorado. Historically, the distribution of the three species spanned the Colorado River, Weber River, Bear River, and Snake River drainages, but now occupy less than 50% of their historical range (Bezzerides and Bestgen 2002). In response to these declines, the Colorado River Fish and Wildlife Council (2004) established a multi-agency agreement to implement conservation measures across the states where the three species exist. Current research identifies non-native species interactions, habitat fragmentation and degradation, and altered thermal regimes as main drivers of observed population declines (Bezzerides and Bestgen 2002; Compton et al. 2008; Jones and Petreman 2013).

In lotic systems where the three species persist, the larvae stage is highly vulnerable to environmental stressors. This heightened vulnerability stems largely from 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 face intense water management pressures. Observed declines in average annual stream flows have led to elevated water temperatures (Reynolds et al. 2015). Furthermore, dams have impacted western stream thermal regimes; reservoir releases and altered flows have caused unnatural increases and decreases in water temperatures (Bezzerides and Bestgen 2002; Dibble et al. 2021). Droughts are predicted to become more frequent, further increasing water temperatures and reducing water availability (Udall and Overpeck 2017). Under these conditions, larval fish may be subjected to extreme temperature conditions, negatively affecting growth and survival (Keller and Klein-Macphee

2000; Jeffries et al. 2016). As these abiotic stressors continue to disturb aquatic systems, defining temperature tolerance ranges for the three species is critically needed.

The purpose of our study was to conduct laboratory-based CT and ILT trials in the field on field-caught larval fish, utilizing their natal stream water and performing the trials in the field. This approach allowed us to validate field-based CT and ILT trials and establish biologically appropriate temperature standards for the three species at the larval life stage. Our study aims to provide valuable information for the conservation of the three species by allowing resource management agencies to identify thermal regimes and corresponding impacts that may negatively affect larval survival and recruitment. This report covers the methodology used in our study, preliminary project results encompassing both years of the project, and a brief overview of future work related to the project.

6.2 Methods

The study was conducted in Roubideau Creek, a tributary of the Gunnison River located near Delta, Colorado, USA. Fish used during the experiments were collected from Roubideau Creek and its ephemeral tributary, Potter Creek (Figure 6.1). The sites were chosen based on historical data documenting consistent and extensive spawning migrations of the three species (Hooley-Underwood et al. 2019).

A mobile laboratory was located at two different sites along Roubideau Creek (Figure 6.1). The specific sites were selected for their accessibility, larval fish availability, and presence of pools sufficiently deep for submersible pumps. In the first year, the laboratory was located near the confluence of Potter Creek, approximately 17.7 river kilometers (rkm) upstream from Roubideau Creek's confluence with the Gunnison River. In the second year, the mobile laboratory was stationed 14.3 rkm upstream from the Gunnison River. Experiments in both years took place from May to June, coinciding with the peak larval rearing period for the three species.

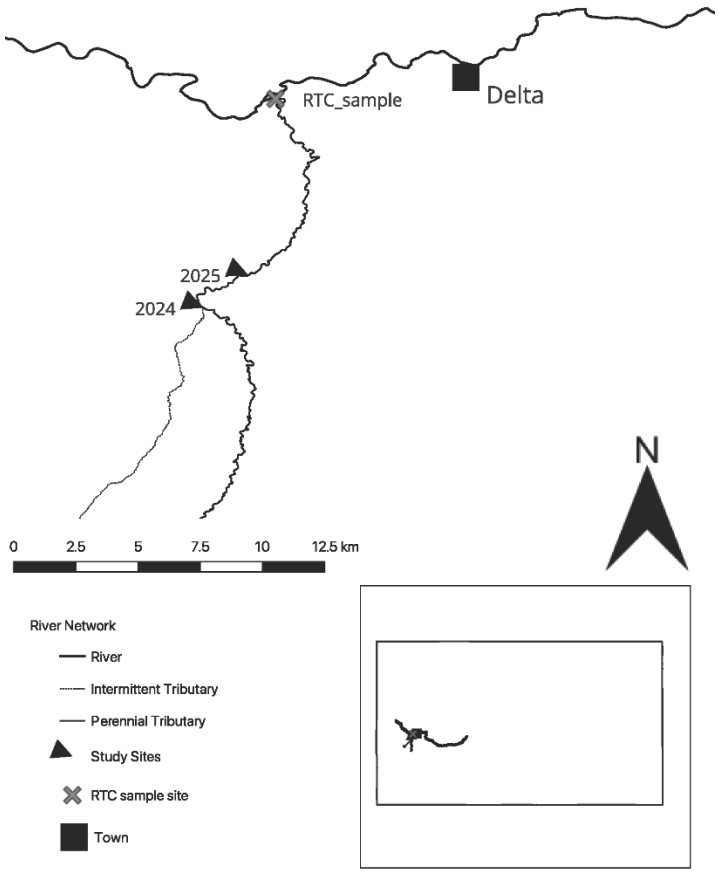

Figure 6.1 Location of study site for three species temperature tests near Delta, Colorado (2024-2025). The rectangle on the inset map displays the site's location in relation to the state of Colorado.

The system's hydrology is primarily driven by spring snowmelt runoff, typically occurring from April to June (Regonda et al. 2006; Bonjour et al. 2023). The surrounding land use is dominated by irrigated agriculture, which requires substantial surface water diversion for crops (e.g., corn, alfalfa), and grazing pastures (Watts 2008; USDA 2022). Water quality in this area is influenced by the dissolution of underlying bedrock, which releases elements such as selenium, nitrate, and sulfate into the surface and groundwater (Mast 2021).

The mobile laboratory was constructed within a 16-foot enclosed trailer to conduct streamside temperature tolerance tests, including upper incipient lethal temperature tests (UILT), critical thermal maximum tests (CTMax), and critical thermal minimum tests (CTMin). Details on the water delivery system, temperature regulation, and interior setup are provided in the sections below.

6.2.1 Weekly Average Temperature (WAT)

To reflect the larvae's recent thermal history, we defined temperature treatments using the weekly average temperature (WAT) of the stream one week prior to experiments. Stream temperature was recorded every 30 minutes using a HOBO logger positioned near the larval

sampling locations. The WAT was calculated as the arithmetic mean of all 30-minute temperature measurements recorded over a given seven-day period.

Laboratory studies involving UILT and CT experiments typically acclimate test subjects to a fixed, manipulated temperature prior to trials. However, we did not use a standard static acclimation protocol. Instead, we used the calculated WAT to categorize the larvae's recent temperature exposure, allowing them to naturally shift their physiology according to the prevailing thermal conditions of the stream. This approach provided an estimate for their innate temperature tolerance and allowed for the observation of any natural physiological changes occurring throughout the three species' larval rearing season.

6.2.2 Larval Collection

Larval Bluehead and Flannelmouth Sucker were collected during May and June 2024 and 2025. Sampling was concentrated in Potter Creek, with additional collections downstream of its confluence with Roubideau Creek. Larvae were collected from low-velocity, bankside habitats using dip nets (47 x 28 cm net opening; 38 cm in depth). Following collection, larvae were transported to the lab in buckets and immediately transferred to extra tanks in the mobile laboratory containing continuous stream water. Larvae were held for a minimum of 24 hours prior to experimentation to reduce handling stress. During the holding period, larvae were fed a combination of 1 mL *Artemia salina nauplii* and crushed *Gammarus* sp. daily. *Artemia salina nauplii* were hatched in a conical tube with aerated salt water (25 ppt) at approximately 26ºC for 24 hours. Filters on the tanks were small enough to not allow the feed to leave the tanks.

6.2.3 Critical Thermal Trials (CT)

The CTMax and CTMin trials were conducted following similar protocols to those described by Bennett and Beitinger (1997). Prior to the trial, larval fish were held in a conditioning tank for a minimum of 24 hours in stream water. A single larval fish was placed into the experimental CT tank. The starting temperature for all trials was the WAT recorded the week before the experiment. Water temperature was increased at a rate of +0.3ºC/minute for CTMax trials using a temperature controller and submersible aquarium heater. Water temperature was decreased by -0.3ºC/minute for CTMin trials using a temperature controller and a steel radiator pipe circulating chilled water. Aeration was continuously supplied to all tanks via a Tetra Whisper air pump and air stones to maintain proper DO levels and ensure a homogenous thermal profile. For CTMin trials, stir plates and bars were also used to improve thermal mixing. Adequate mixing CTMax trials was achieved by the large surface area of the submersible heater and the mixing provided by the incoming aeration.

The endpoint for all trials was loss of equilibrium (LOE), defined as the failure to maintain dorsal-ventral orientation (Bennett and Beitinger 1997). Upon observing LOE, the larvae was immediately transferred to a recovery tank at the original WAT for 20 minutes and monitored for post-trial mortality (Cadmus et al. 2014). Since critical thermal trials are not intended to be lethal, any larval fish that died during the trial or the subsequent recovery period was excluded from data analyses (Beitinger et al. 2000). Initial and final temperatures were recorded using calibrated ThermPro thermometers. Dissolved oxygen and pH were measured with YSI ProODO and pH instruments, respectively. At the conclusion of all experiments, larvae were euthanized with MS-222, measured for standard length (mm), and preserved in 95% EtOH in a 2 mL microcentrifuge tube subsequent for identification.

6.2.4 Incipient Lethal Trials (ILT)

Upper incipient lethal tests were only conducted in 2025, resulting in three complete 7-day trials. The experiment utilized a total of four temperature treatments: the control, two UILT treatments with increased temperatures from the WAT, and one stream treatment (i.e., natural fluctuation). The experimental setup consisted of racks where individual rows were assigned to a temperature treatment. Each treatment row contained seven 2 L polyethylene tanks (Iwaki Aquatics), which served as replicates for the UILT trials. For the first week, the control row used four tanks, increasing to seven control tanks for subsequent trials. The four temperature regimes included: a control which was maintained at the WAT, two UILT treatments designed to test increased thermal stress, and a stream row. The stream row was conducted on a separate rack, exposing larvae to the natural, fluctuating temperature regime of Roubideau Creek to assess mortality to *in situ* conditions. No water entering or leaving the trailer that was pumped from Roubideau Creek was manipulated in any way. The UILT temperatures were based on 2025 temperature data from Roubideau Creek. The standard deviation (SD) of the creek's water temperature during the larval rearing season was calculated to be 3ºC. This value was used to define the two UILT treatments. The first treatment was set at + 2x SD +6ºC relative to the WAT (WAT + 2xSD). This temperature encompassed the typical, high-end thermal magnitude experienced by larvae in the creek. The second treatment was set at +12ºC above the WAT, representing an extreme temperature stress beyond the normal range. In the final week of testing, the two temperature treatments were adjusted +12ºC and +15ºC relative to the WAT to avoid treatment overlap from the previous tests.

Prior to the start of a test, larvae were grouped into cohorts of ten fish using a fine-mesh aquarium net (8 cm x 10 cm). Each cohort was placed into a translucent container before being transferred into their assigned UILT or stream tanks. Once the assigned tank temperature was stable, the ten larvae were transferred directly into the tank. Larvae were observed for the first 60 minutes post-transfer to monitor for immediate mortality. If a mortality occurred within this initial hour, the deceased individual was removed and replaced with a new larva; the dead larvae was excluded from the data analysis. Subsequent observations were made every eight hours for the remainder of the 7-day (168 hour) test duration. Upon observation of mortality, the individual was immediately removed from the tank and preserved in a 2 mL microcentrifuge tube filled with 95% EtOH for post-test identification.

During each observation check, the temperature of every tank was recorded using calibrated ThermPro thermometers. To document fine-scale temperature variation within each UILT row, a Pro v2 HOBO logger was placed into a tank within each row to record temperature data every 30 minutes throughout trials. Dissolved oxygen and pH were recorded from the first and last tank in each row at every check using YSI ProODO and pH instruments, respectively. All larvae were once daily with approximately 1 mL of a combination of *Artemia salina napulii* and *Gammmarus* sp. At the conclusion of the 7-day trial, all surviving larvae were euthanized with tricaine methanolsulfate buffered with sodium bicarbonate (MS-222; Western Chemicals). The standard length of each individual was measured (mm), and the fish were preserved in 2 mL microcentrifuge tubes with 95% EtOH for fish identification.

6.2.5 Water Delivery System and Temperature Regulation

The mobile laboratory was designed to operate as a flow-through and recirculating system to deliver water from Roubideau Creek (Figure 6.2). A submersible pump (Dewalt ¾ HP; 12.5 m head pressure at 314 L/min) transferred creek water through a 100µm stainless mesh screen filter into three insulated storage tanks (475 L) located outside of the trailer. Two tanks were designated for cooling and one for heating to achieve the upper incipient lethal temperatures treatments. The primary cold storage tank was equipped with a Frigid Units immersion chiller, precisely set to maintain a temperature 5ºC below the coldest UILT treatment. A secondary cold buffer tank, connected to two ½ HP Pod Chillers (The Pod Company, Dover, DE) set to approximately 2ºC, served as a constant cold water supply for the primary tank, enhancing temperature stability. The hot storage tank was heated to a temperature 6ºC above the warmest UILT treatment maximum using four Process Technology submersible heaters. To ensure dissolved oxygen was not a limiting factor for larval survival, it was supplemented in each primary storage tank using a Crystal Clear pond aerator (0.5 m3/min airflow). Storage tank temperatures were established the night prior to an ILT trial, requiring eight hours to reach desired set points. Once stable, water was pumped from each primary storage tank into the internal manifold system using dedicated submersible utility pumps (Everbilt 1/6 HP; 7.6 m head pressure at 76.5 L/min). The manifold system was constructed out of ¾" PVC pipe and routed the hot and cold water supplies to mix into individual 15.15 L head tanks for each row. Individual ILT tank temperature were allowed six hours to stabilize before the transfer of larvae, ensuring the desired static temperature treatments were consistently maintained.

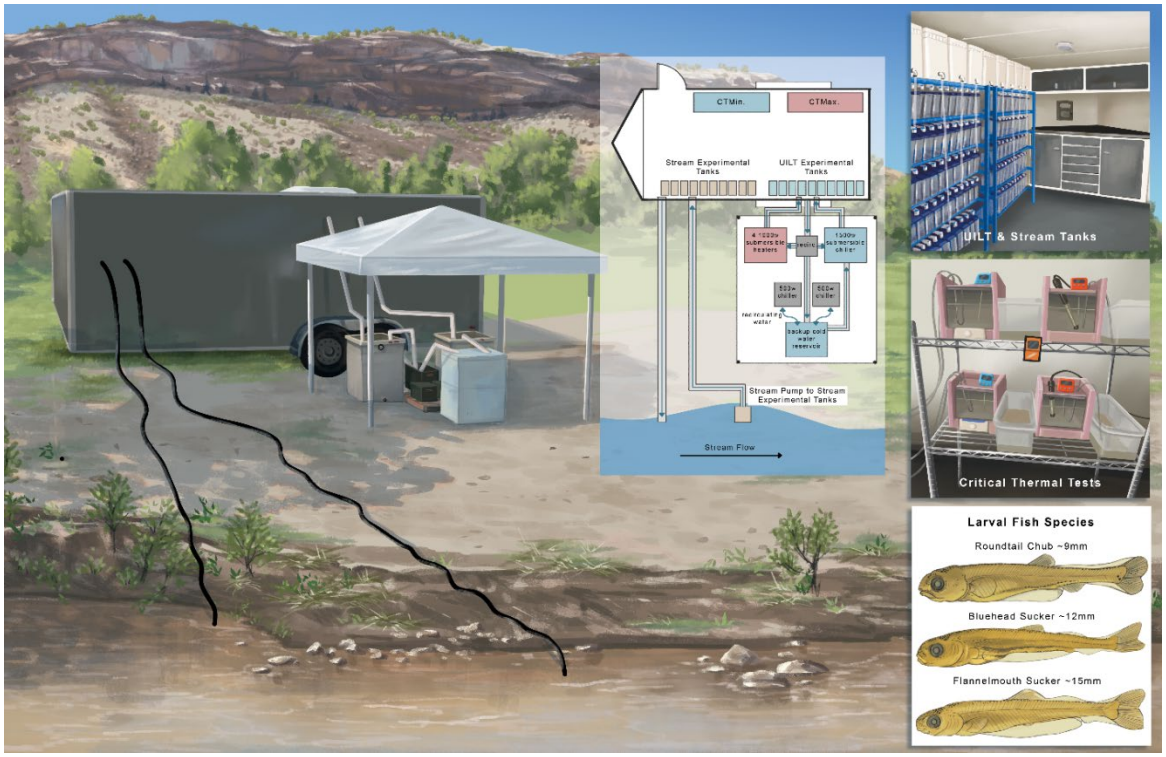

Figure 6.2 Mobile laboratory deployed at Roubideau Creek for temperature research. Inserts illustrate the trailer's water flow, storage, stream water intake, the upper incipient lethal test and stream test setup, the critical thermal test apparatus, and the three larval fish species studied; Roundtail Chub, Bluehead Sucker, and Flannelmouth Sucker. (Illustration by Jennifer Clausen, www.jacdraws.com)

In addition to the UILT experiments, the mobile laboratory's plumbing incorporated a bypass to receive direct stream water, enabling the study of larval survival under natural temperature fluctuations (referred to as the stream ILT treatment). This direct connection also functioned to hold larvae prior to trials in a separate row from the stream treatment row. For the stream ILT treatment, a direct line was established from the creek to the head tanks using a second submersible pump (Dewalt ¾ HP; 12.5 m head pressure at 314 L/min). This system maintained a continuous flow rate of 222.6 mL/min (SD: 102.2 mL/min) into the tanks. All outflow from the stream ILT rack system was discharged directly back into Roubideau Creek, functioning as an open-flow-through system as no physical or chemical manipulation of the water occurred within the stream ILT system, which allowed for the direct discharge.

6.2.6 Interior Setup for ILT Experiments

The experimental UILT tanks inside the mobile laboratory were arranged on two custom Iwaki Aquatic Rack systems (Holliston, MA). One rack was dedicated to the temperature-manipulated UILT treatments, while the second rack housed the stream ILT treatments and held the holding tanks. Each rack contained five rows, each containing ten 2 L experimental tanks, resulting in 50 total tanks per rack. Each row served as a single temperature treatment, with the ten tanks within that row functioning as an experimental replicate (n = 10). For UILT trials, each row's water supply originated from an overhead manifold and were gravity-fed into a corresponding head tank. Solenoid valves (REDHAT model 8210G095; normally closed) were positioned on the cold-water manifold and were controlled by temperature controllers (B-series Love Controls Division; model 16B). These controllers activated the solenoid valves to release cold water into the head tank when its temperature exceeded a set threshold of 0.2ºC above the target temperature treatment. The hot manifold was not regulated by solenoid valves, allowing for constant flow of hot water into the designated head tanks. Water leaving the UILT tank rack was returned via recirculation to either the hot storage tank or the cold buffer storage tank (Figure 6.2). This recirculating system was implemented to reduce the energy demand for cooling and heating, to minimize sedimentation within the system, and eliminate the need for discharge permits. Sedimentation was a key concern because excessive particulate matter would reduce visibility of larvae for observation and could obstruct flow throughout the system. The high turbidity potential of Roubideau Creek, a plateau stream often experiencing extreme conditions after precipitation, necessitated this design.

6.2.7 Water Volume of System and Power Requirements

The UILT system maintained a total water volume of 803 L, which was distributed among its components: 303.3 L in the hot storage tank, 286 L in the cold storage tank, 171.7 L in the cold buffer tank, and 42 L within 21 2 L UILT tanks. Not all 50 tanks in the racks were used, only three rows and seven tanks in each row. To maintain system balance and introduce fresh water, approximately 57 L of fresh stream water was added daily, accounting for water lost to evaporation. Water levels in the storage tanks were regulated by float switches (SJE-Rhombus), which activated a submersible pump (Danner; 3 m head pressure at 28.4 L/min) when the hot or cold storage tank levels dropped to 234.6 L and 205.9 L, respectively. Specifically, the pump transferred water from the outflow recirculation bin to the hot storage tank to the cold storage tank. Synchronously, the cold buffer storage tank was also replenished from the outflow recirculation bin upon the trigger of the primary float switch, ensuring a continuous supply of cold water. The entire laboratory setup, including all experimental equipment, required a

substantial 11,129 running watts of power, which was supplied by a rotating sequence of three grounded gas generators.

6.2.8 Larval Fish Species and Stage Identification

Following UILT and CT experiments, all larvae were identified to species and developmental stage using stereo microscopes, based on established protocols by Snyder et al. (2004; 2016). Larval development was broadly classified into three stages: protolarvae, mesolarvae, and metalarvae. This staging was defined by the progression of median finfold absorption, adult fin ray development, and gut differentiation. All larvae collected fell into the mesolarvae and metalarvae stages, with mesolarvae further categorized as preflexion (straight notochord) or postflexion (upward-flexed notochord). Standard length (mm) and vent position served as the initial diagnostics for separating the two main families. Cyprinid larvae were generally <10 mm in the mesolarvae stage with an anterior vent located near the dorsal fin, while Catostomid larvae were 12 mm+ with a more posterior vent located near the caudal fin (Figure 6.2) Within Catostomidae (suckers), species identification relied on a combination of size at development and chromatophore distribution (i.e., pigment patterns). Flannelmouth Suckers were identified by being approximately 2 mm larger than Bluehead Suckers at the same developmental stage, exhibiting sparse ventral pigmentation (≤ 7 melanophores), and an organized "herringbone" dorsal chromatophore pattern, contrasting with the Bluehead Sucker's interrupted ventral pattern (> 7 melanophores) and densely scattered dorsal chromatophore distribution. White Sucker larvae *Catostomus commersoni* were also present in Roubideau Creek, and were identified by a distinct, continuous ventral line of melanophores. For Catostomid metalarvae, species determination shifted from pigmentation to size relative to the onset of pelvic buds/fins and gut loop formations, which develop at smaller sizes in Bluehead Suckers compared to Flannelmouth Suckers. Hybrids, including crosses between the native suckers (Bluehead x Flannelmouth) and the White Sucker, were identified by exhibiting intermediate characteristics across the mentioned diagnostic features. The Roundtail Chub (Cyprinidae) was distinguished from non-test Cyprinids by its consistently larger size at less advanced developmental stages, supplemented by a key meristic count: Roundtail Chub possessed 43+ myomeres, differentiating them from the <40 count observed in dace and minnows. To ensure the accuracy and reliability of all classifications, a quality control protocol was implemented where all larvae were identified and staged by two blind readers, with a third identifier used to resolve any disagreements, and only fish with species and stage agreement between two readers were considered accurately identified.

6.2.9 Water Chemistry

To assess potential environmental influences on temperature tolerance, water samples were collected and analyzed for heavy and trace metals (e.g., aluminum, arsenic, cadmium, copper, iron, magnesium, lead, selenium, zinc, calcium, potassium, manganese, and sodium) and key nutrients (e.g., phosphorus and chloride). For metal analysis, two 60 mL samples were collected weekly from Roubideau Creek, Potter Creek, and the storage tanks. The first 60 mL aliquot was retained for total metal analysis, while the second was filtered through a 45 µm filter for dissolved metal analysis. Both metal samples were preserved with six drops of 3% high-purity nitric acid (HNO_3). For nutrient analysis, two non-filtered water samples were collected in 15 mL Falcon tubes and immediately placed on ice, with one of the two nutrient samples chemically preserved with 3% sulfuric acid (H_2SO_4). All samples were submitted to the Colorado Parka and

Wildlife Toxicology and River Watch Laboratory for analysis, which was completed within six months of collection for metals and 30 days for nutrients.

6.2.10 Statistical Analyses

6.2.10.1 Incipient Lethal Trials

Statistical analysis for the ILT, encompassing both the UILT and stream experiments, were structured to assess both time-dependent survival probabilities and endpoint survival rates. The time-dependent probability of larval survival over the seven-day (168-hr) trial for the fish species was estimated using the Kaplan-Meier survival model (Therneau 2024). Differences in survival probabilities between the various temperature treatments were assessed using a log-rank test, with statistical significance was set at 0.05 (α). Only Bluehead Sucker and Flannelmouth Sucker data were evaluated as no Roundtail Chubs were collected for ILT experiments. To compare the final percent of larval survival among the UILT and stream ILT temperature treatments, a binomial generalized linear model (glm) was fit to data using the glm() function from the R stats package. Following model fitting, an analysis of variance (ANOVA) designed for unbalanced glms was applied ($\alpha = 0.05$). If significant differences were detected in the ANOVA results, Tukey's Honest Significance test was used for post-hoc multiple comparisons. It is important to note that the UILT data analyzed with separate weekly cohorts (by WAT), while the stream ILT results were evaluated collectively as a single dataset. All analyses were conducted in the R programming environment (version 4.5.0) utilizing the survival and surv miner packages.

6.2.10.2 Critical Thermal Trials

Statistical analyses for the CT experiments were used to evaluate changes in thermal tolerance across larval development and to compare responses between species. To detect if the mean LOE varied across the WATs for the Bluehead Sucker and Flannelmouth Sucker, separate one-way ANOVA with an unbalanced design were conducted for each species. Statistical significance for all ANOVA analyses was set at $\alpha = 0.05$. If significant differences were detected across WATs, then Tukey's Honest significance test was used for post-hoc analysis. Additionally, simple linear regression (SLR) models were fit to the CTMax and CTMin values to evaluate the linear association between the WAT and mean LOE. Separate SLR models were developed and evaluated for the Bluehead Sucker and Flannelmouth Sucker. Prior to fitting these regression models, the underlying statistical assumptions of normality and equal variance were visually assessed through QQplots and Residual vs Fitted plots. Respectively. A regression model could not be applied to Roundtail Chub as LOE data was collected for only a single WAT. Finally, the CTMax and CTMin data of the two suckers were compared using a two-way analysis of covariance (ANCOVA), treating species as an interactive effect to evaluate whether upper and lower temperature tolerances were significantly different between the two larval species. All statistical analyses for CTMax and CTMin were conducted in R (version 4.5.0).

6.3 Results

During the larval rearing seasons (May and June) of 2024 and 2025, Roubideau Creek experienced temperatures spanning from as low as 5ºC to a maximum of 29.5ºC. The stream showed diel temperature variability, with fluctuations averaging 8.8ºC ± 2.0 and reaching a maximum daily range of 12.3°C. The emergence of Catostomid larvae (Bluehead and Flannelmouth Suckers) was first detected around mid-May both years, typically when the WAT reached approximately 14-16ºC, which aligns with previous reports for these species in the White River (Fraser et al. 2019). Roundtail Chub larvae were observed approximately one month later in June, coinciding with an increased WAT of approximately 21.5ºC. In total, the 2025 field season generated three weekly average temperatures used in UILT trials and six for CT trials (Figure 6.3, Table 6.1), while two weekly average temperatures from the 2024 field season were incorporated into the CT trials (Cadmus et al. 2024). It is noted that the first week of WAT of the 2025 season (13.8°C) was calculated using only a three-day average, as larval emergence occurred prior to the completion of seven full days of temperature data collection in Roubideau Creek.

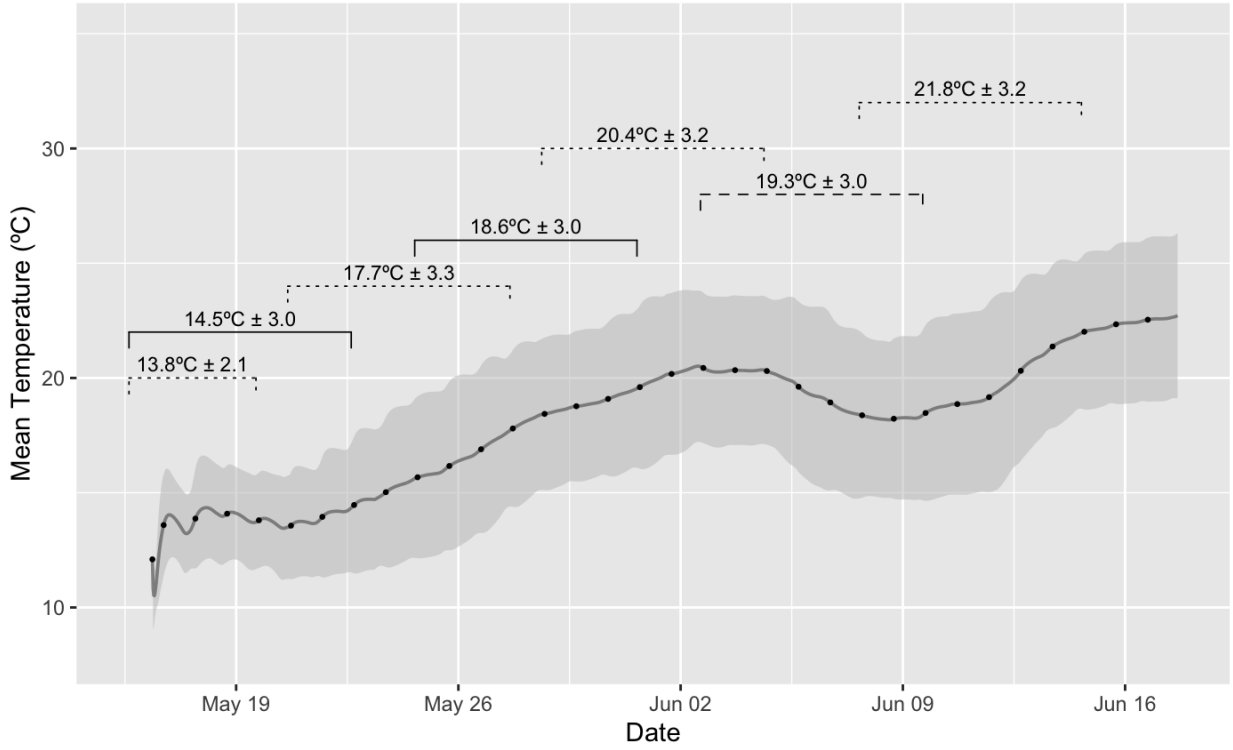

Figure 6.3 2025 temperature (ºC) data for Roubideau Creek from May through the end of the field experiments in June. Temperature is shown as a seven-day rolling average, with the shaded region displaying the corresponding standard deviation. Weekly average temperatures (WAT; with standard deviation) used in trials are annotated as brackets. Bracket notation relating to WAT used on experiment type is as follows: CT - - - ; UILT —— ; CT + UILT – – –.

Table 6.1 Minimum and maximum observed temperatures for each of the Weekly Average Temperatures (WAT) used for critical thermal trials (CT) and upper incipient lethal trials (UILT) collected from 2025 field season.

WAT (°C)	Maximum Observed (°C)	Minimum Observed (°C)	Trial Type
13.8	18.7	10.7	CT
14.5	23.2	9.2	CT & UILT
17.7	24.5	11.6	CT
18.6	27.8	11.4	CT & UILT
19.3	26.5	15.2	UILT
20.4	28.0	15.4	CT
21.8	27.8	16.4	CT

6.3.1 Water Chemistry

Water samples were collected and analyzed from three locations; Roubideau Creek, Potter Creek, and the storage tanks on three different occasions in 2025. Water hardness, calculated from total calcium and magnesium, varied significantly across these locations: 144-188 mg/L $CaCO_3$ in Roubideau Creek, 341-440 mg/L $CaCO_3$ Potter Creek, and 135-222 mg/L $CaCO_3$ inside the storage tank. Metal analysis showed that the acute and chronic water quality standards were not exceeded in Roubideau Creek or Potter Creek. However, elevated levels of dissolved copper were detected in the storage tank on two separate sampling events. On the first sampling event, dissolved copper exceeded the Colorado Department of Public Health and Environment (CDPHE) acute standard, measuring 22.83 µg/L when the water hardness was 135 mg/L $CaCO_3$. On the third sampling event, dissolved copper exceeded the CDPHE chronic standard, recorded at 19.37 µg/L $CaCO_3$ when the water hardness was 219 mg/L $CaCO_3$. The occurrence of elevated copper levels was likely a result of the Frigid Units submersible chiller or POD chillers, which use copper coils for heat exchange inside the cold storage tanks. Comprehensive water chemistry results, including all metal and nutrient concentrations are presented in Table 6.2.

Table 6.2 Results of Roubideau Creek, Potter Creek, and storage tank dissolved and total metal concentrations using ICP-OES method for May 24th, June 1st, and June 12th, 2025. Concentrations are listed as mg/L. No concentrations are listed for detections less than the method detection limit (<MDL). Asterisks (* *) around individual numbers signify exceedance of chronic standards, and bold numbers signify exceedance of acute standards set by the Colorado Department of Public Health and Environment.

Date	May 24		June 1		May 24		June 1		June 12		May 24		June 1		June 12	
Location	Roubideau Creek				Potter Creek						Storage Tank					
Element	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Dissolved
Al	348.0	16.89	202.5	11.58	29.41	9.974	76.67	12.93	443.7	20.08	248.2	7.062	124.9	8.602	31.35	13.18
As	0.9413	0.9969	1.522	1.431	0.7772	0.5503	1.105	1.108	1.743	1.502	<MDL	1.283	1.197	<MDL	<MDL	1.112
Ca	43120	43080	53480	56900	860850	82580	101500	103400	101400	92650	41800	39850	48780	50380	66820	65390
Cd	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Cu	1.409	1.040	1.151	1.280	0.965	0.7730	1.028	0.9813	10.33	9.348	38.17	*22.83*	23.04	12.62	25.06	*19.37*
Fe	247.0	1.748	142.0	2.06	85.38	1.553	132.8	10.03	279.3	<MDL	204.4	<MDL	88.86	<MDL	4.530	<MDL
K	2407	2322	2674	2763	5640	5680	7321	7244	9516	8977	2505	2446	2960	2874	3932	3930
Mg	8909	8958	10510	11180	34020	32740	43720	44060	41480	38440	8996	8546	10240	10520	13470	13490
Mn	16.78	6.386	12.96	6.705	26.17	23.17	45.80	46.58	42.66	22.13	15.96	9.39	12.34	7.410	6.748	5.776
Na	33590	33450	49240	50930	57840	59110	90630	88690	88130	83380	28870	29340	40810	40540	52040	53770
Pb	<MDL	<MDL	<MDL	<MDL	0.4817	0.2125	0.2982	0.7773	1.132	0.5517	2.438	<MDL	1.340	<MDL	<MDL	<MDL
Se	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Zn	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	2115	1539	2197	01105	1230	718.7
Hardness	144	144	177	188	354	341	433	440	424	390	141	135	164	169	222	219

6.3.2 Incipient Lethal Trials

A total of 870 larvae were collected and used for ILT trials. Out of the 870 larvae, 835 larvae were identified to the family level as Catostomidae. For the purpose of estimating overall survival and mortality rates in the ILT trials in this report, all Catostomid larvae are pooled. Throughout the duration of the seven-day trials, the water quality parameters were maintained within acceptable limits: DO levels ranged from 6.4-12.5 mg/L, and pH ranged from 8.25-8.53. Critically, the UILT system maintained temperature stability for the full seven-day duration of each trial, with only minor variation (Table 6.3).

Table 6.3 Upper incipient lethal test temperatures throughout the experiments and key trial results (e.g., LT_{50} in hours) for 2025 field season. Dashes indicate treatments where LT_{50} was not reached.

WAT (°C)	Time	Treatment (°C)	Sample Size	Time to LT_{50}
14.5	Week 1: 5/23-5/30	14.5 ± 0.8	40	40
		20.5 ± 0.8	69	-
		26.5 ± 0.6	69	-
18.6	Week 2: 6/02-6/09	18.6 ± 0.5	69	-
		24.6 ± 0.4	70	-
		30.6 ± 0.6	68	-
19.3	Week 3: 6/10-6/17	19.3 ± 0.8	64	-
		31.3 ± 0.6	68	8
		34.3 ± 0.8	67	4

Differences in the proportion of survival at the 168-hr endpoint were detected within each week UILT trial (Figure 6.4). Across all weeks, the highest overall survival proportions were observed in the 24.6ºC and 26.5ºC treatments, while the lowest proportions were detected at 31.3ºC and 34.3ºC. In week one, multiple comparisons revealed that only the 14.5ºC differed from both the 20.5ºC and 26.5ºC treatments (Figure 6.4A; $X^2_{2,18} = 37.5$, $p < 0.05$). In week two, differences in survival proportion were only detected between the24.6 and 30.6ºC treatments (Figure 6.4B; $X^2_{2,21} = 13.0$, $p < 0.05$). In the third week, a significant difference in the proportion of survival was detected between all temperature treatments (Figure 6.4C; $X^2_{2,21} = 142.7$, $p < 0.05$).

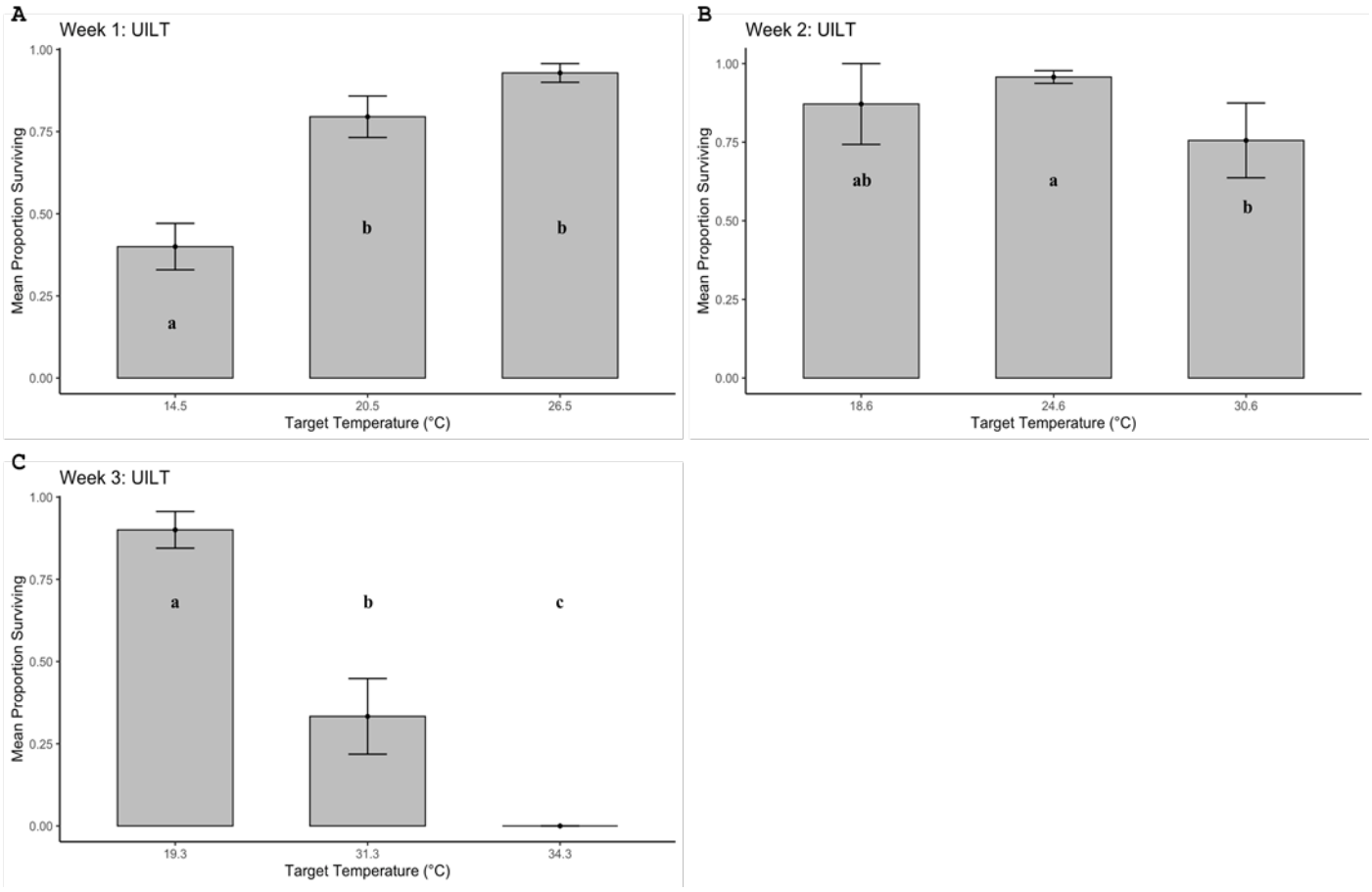

Figure 6.4 Barplots representing mean proportion of larval survival by temperature treatment across UILT trials in 2025 across the three weeks. Letters within the barplot represent results of multiple comparison test. Matching letters indicate non-significant relationship between two or more treatments ($p < 0.05$).

The Kaplan-Meier survival analysis demonstrated significant differences in survival curves across the temperature treatments for all three weekly UILTs (Figure 6.5). In week one, a significant difference was detected among temperature treatments (Figure 6.5A; $X^2_{2,178} = 39.8$, $p < 0.05$). The estimated probability of survival at 168 hours was highest at 26.5°C (0.93 ± 0.03), followed by 20.5°C (0.81 ± 0.06), and lowest at 14.5°C (0.40 ± 0.19). The 14.5°C treatment was the only one in week one to reach the LT_{50} (50% lethal temperature), which occurred at 40 hours. Survival curves in the second week were significantly different across treatments (Figure 6.5B; $X^2_{2,207} = 13$, $p < 0.05$, $_{2,207} = 13$). The 168-hr estimated survival probabilities were 0.87 ± 0.05 at 18.6ºC, 0.96 ± 0.03 at 24.6ºC, and 0.75 ± 0.07 at 30.1ºC. No LT_{50} was reached for any temperature treatment. Highly significant differences were observed across treatments in the third week of UILTs (Figure 6.5C; $X^2_{2,199} = 188$, $p < 0.05$). The 168-hr estimated survival probabilities showed a sharp decline at the highest temperatures: no survival at 34.3°C, 0.32 ± 0.18 at 31.3°C and 0.91 ± 19.3°C. Both high temperature treatments reached the LT_{50}: 31.3ºC at 8 hours and 34.3ºC at 0 hours.

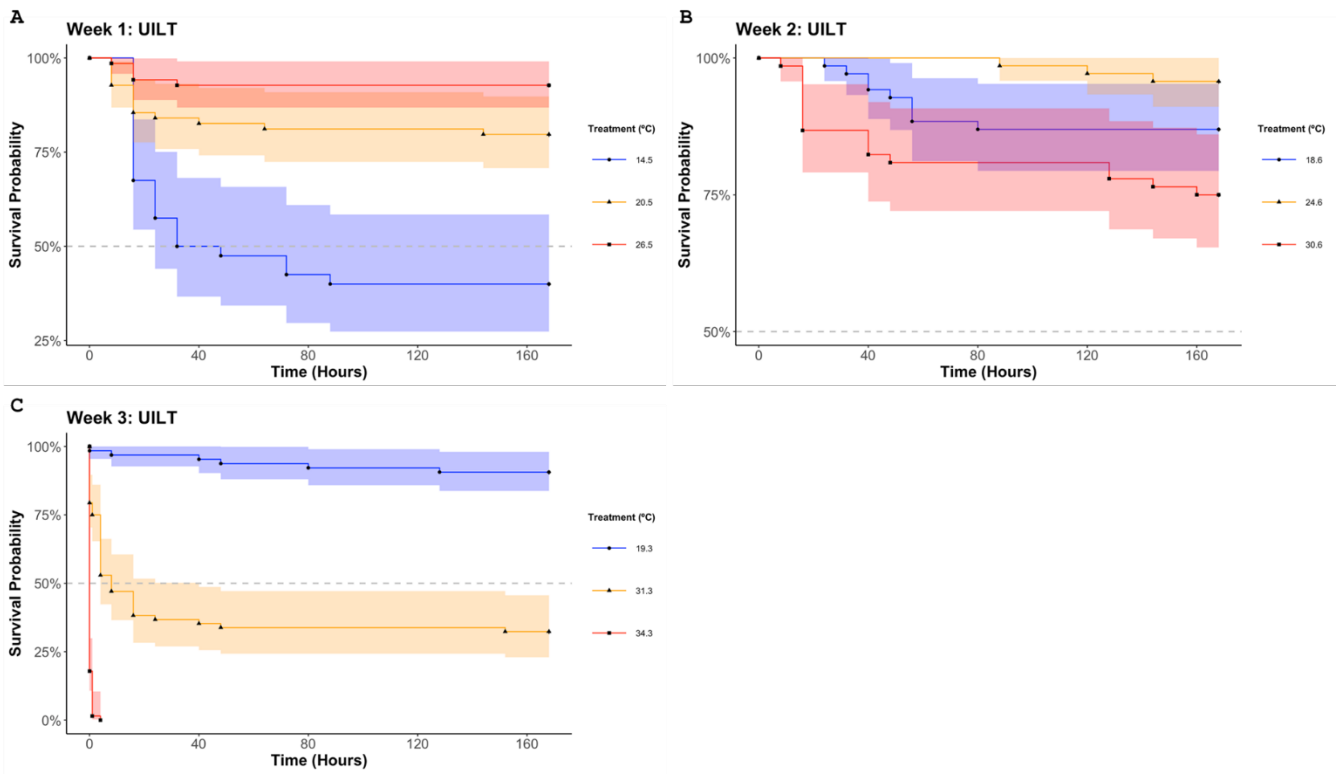

Figure 6.5 Survival probability of Catostomid larvae collected from Roubideau Creek during upper incipient lethal trials (UILT) in 2025, plotted as a function of experimental duration (hours). Different temperature treatments are represented by point types, and the shaded areas represent the 95% confidence intervals of the modeled survival data. A: Week 1 (WAT 14ºC); B: Week 2 (WAT 18.6ºC); C: Week 3 (WAT 19.3ºC). Grey dashed lines represent LT_{50} mortality.

A total of three stream ILT trials were conducted, with one trial occurring during each of the three experimental weeks. Each trial was categorized by the mean temperature observed over the full 168 hours. Temperature conditions in the stream fluctuated across the three weeks. In week one, temperatures ranged from 13.8-27.7ºC (19.2ºC $\pm$3.0). In week two, temperatures ranged from 15.2-26.5ºC (19.5ºC $\pm$ 3.2). In week three, temperatures ranged from 17.7-30.2ºC (23.3ºC $\pm$ 3.7.; Figure 6.6). The Kaplan-Meier survival analysis revealed significant differences among survival curves across stream ILT treatments ($X^2_{2,200} = 123$, $p < 0.05$; Figure 6.7).

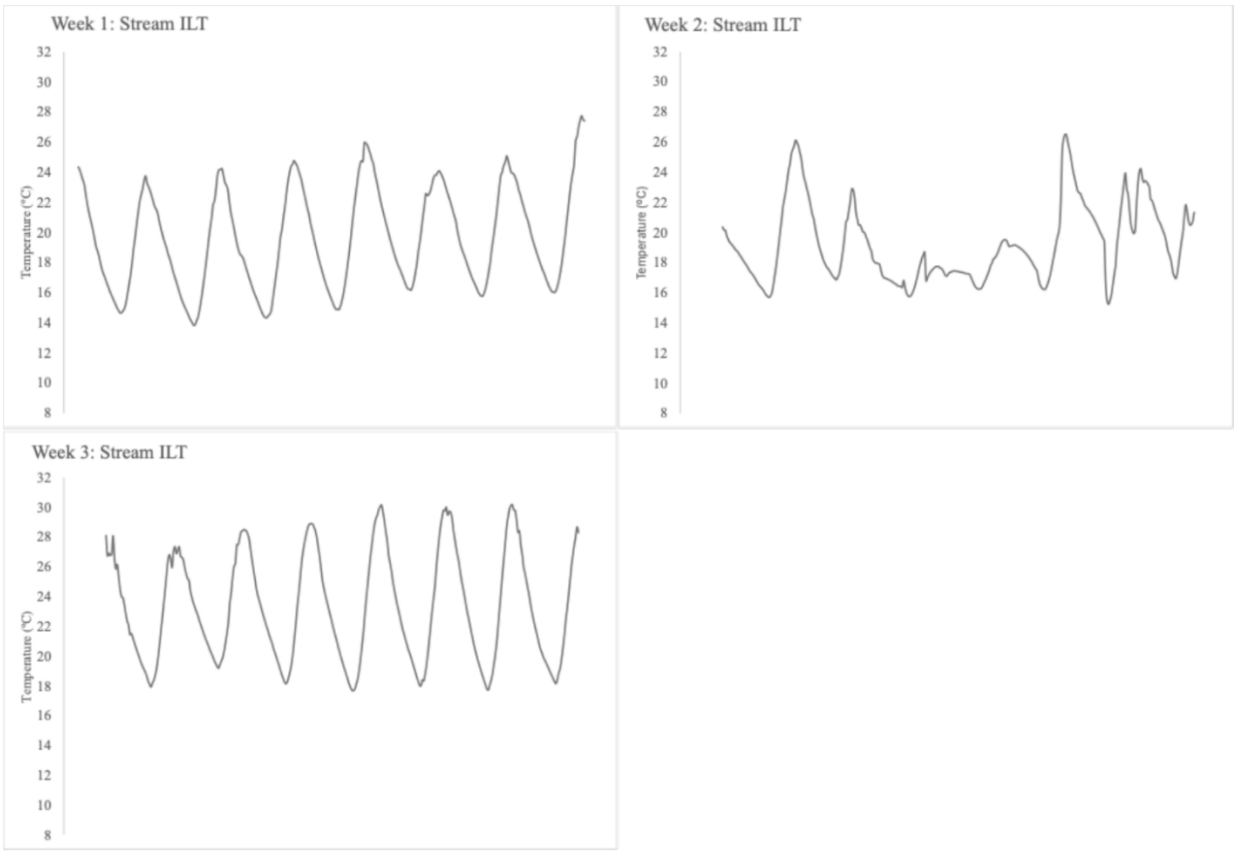

Figure 6.6 2025 temperature plots for stream ILT treatment. Graphs are separated by week of trial, with temperatures recorded by a HOBO logger every 30 minutes placed inside an ILT tank.

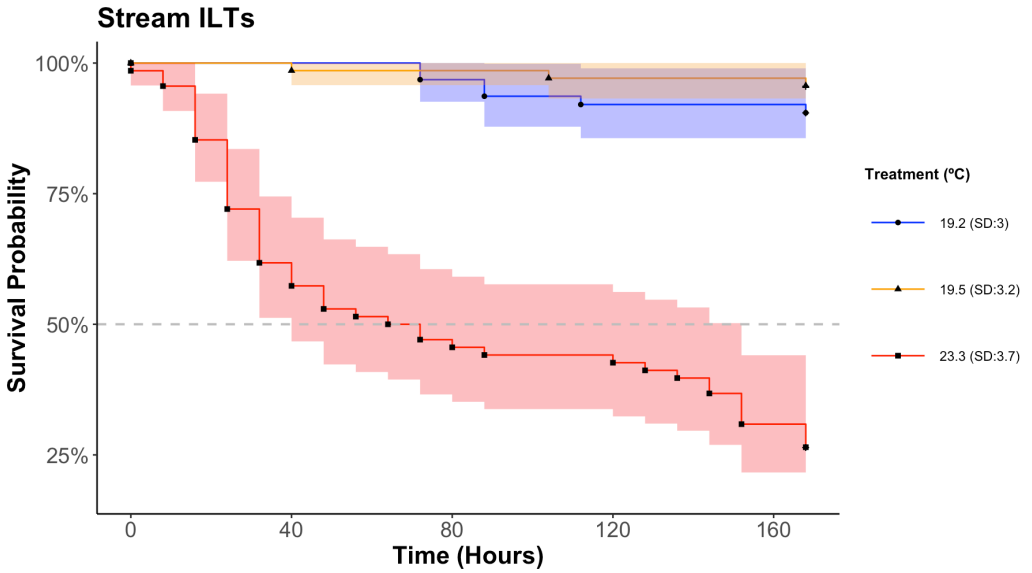

Figure 6.7 Kaplan-Meier survival curves for Catostomid larvae collected in Roubideau Creek that underwent stream ILT trials in 2025 as a function of total hours over the experiment. Different treatments are represented by point type with color-shaded areas illustrating 95% confidence intervals of modeled data. Grey dashed lines represent LT_{50} mortality.

Modeled survival proportion at 168 hrs were high for the first two weeks and dramatically lowering for the third week (week 1: 0.90 ± 0.04, week 2:0.95 ± 0.03, week 3: 0.26 ± 0.20). LT_{50} occurred only during the third week of stream ILTs, at the 23.3ºC mean temperature at 68 hours. When comparing the proportion of survival at the end of the experiment (Figure 6.8), only the third week's stream ILT treatment (23.3 °C mean) was found to be significantly different from the others ($X^2_{2,21} = 99.7$, $p < 0.05$). The survival proportions for the 19.2 and 19.5ºC mean temperature stream treatments were statistically similar.

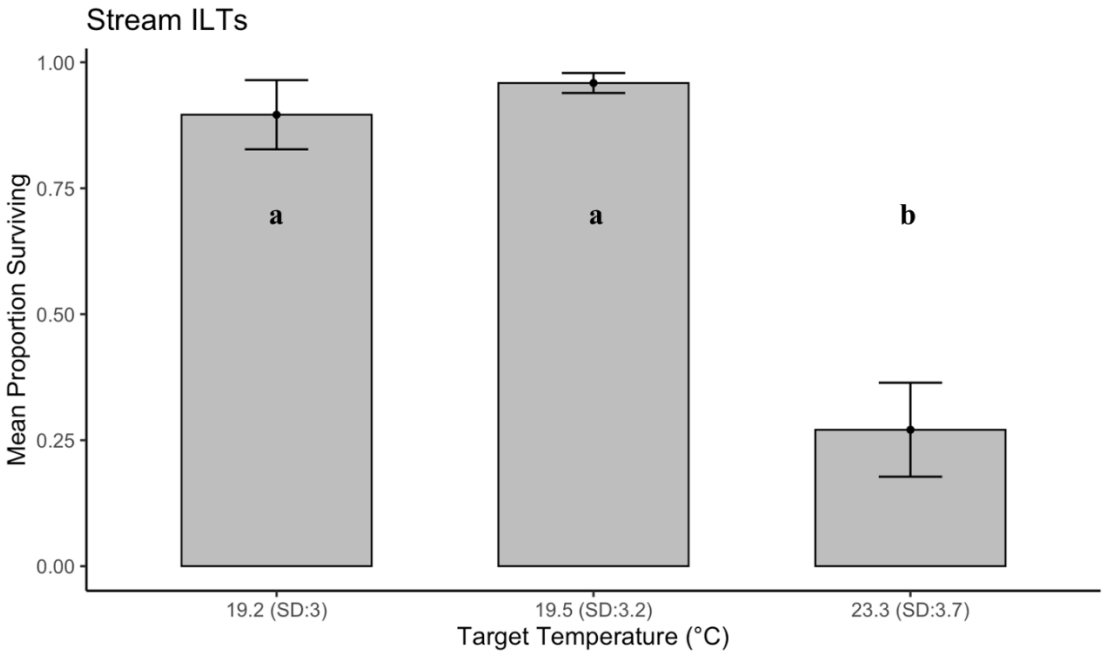

Figure 6.8 Barplots representing mean proportion of larval survival by temperature treatment of the stream ILT trials in 2025. Letters within the barplot represent results of multiple comparison tests. Matching letters indicate non-significant relationship between two or more treatments.

6.3.4 Critical Thermal Trials

Over the 2024 and 2025 field seasons, thermal tolerance was evaluated across nine WATs CTMax and CTMin (Table 6.4). In total, 206 trials were conducted for CTMax and 208 trials for CTMin. Out of those trials, 76 Bluehead Suckers (BHS), 64 Flannelmouth Suckers (FMS), and 29 Roundtail Chub (RTC) had CTMax data collected. For CTMin, 78 Bluehead Suckers (BHS), 63 Flannelmouth Suckers (FMS), and 30 Roundtail Chubs (RTC) were tested (Table 6.4. Throughout the CT trials, environmental conditions remained relatively stable, with DO and pH varying by an average 2.63 ± 1.20 mg/L and 0.24 ± 0.86, respectively. Detailed results for the Roundtail Chub CT trials are presented in the 2024 Water Pollution Studies Annual Report (Cadmus et al. 2024).

Table 6.4. Critical thermal maxima (CTMax; +0.3ºC/min) and minima (CTMin; -0.3ºC/min) estimated from trials with the three species larvae for nine weekly average temperatures (WAT). Standard error and sample size are provided for mean CT estimates. * signifies WAT collected in 2024. BHS: Bluehead Sucker, FMS: Flannelmouth Sucker, RTC: Roundtail Chub

WAT (°C)	Species	CTMin (°C)	CTMax (°C)
13.8	BHS	7.4 ± 0.8 (n=4)	28.5 ± 3.4 (n=2)
	FMS	7.3 ± 0.3 (n = 10)	30.7 ± 0.8 (n = 5)
14.5	BHS	7.3 ± 0.1 (n = 11)	32.8 ± 0.8 (n = 8)
	FMS	7.4 ± 0.1 (n = 14)	32.8 ± 0.7 (n = 18)
16.7*	BHS	7.9 ± 0.4 (n = 9)	33.6 ± 0.6 (n = 10)
	FMS	8.9 ± 0.6 (n = 11)	32.9 ± 0.8 (n = 7)
17.7	BHS	7.5 ± 0.1 (n = 13)	32.7 ± 0.4 (n = 4)
	FMS	7.9 ± 0.4 (n = 9)	33.1ºC ± 0.3 (n = 18)
18.6	BHS	7.4 ± 0.1 (n = 15)	35.2 ± 0.3 (n = 13)
	FMS	7.3 ± 0.1 (n = 7)	35.8 ± 0.3 (n = 6)
20.4	BHS	7.5 ± 0.1 (n = 15)	34.6 ± 0.2 (n = 12)
	FMS	7.7 ± 0.3 (n = 7)	34.4 ± 0.3 (n = 6)
20.9*	BHS	7.7 ± 0.4 (n = 3)	33.9 ± 0.4 (n = 11)
	RTC	7.0 ± 0.1 (n = 30)	33.6 ± 0.2 (n = 29)
21.8	BHS	8.1 ± 0.5 (n = 8)	36.2 ± 0.1 (n = 16)
	FMS	8.4 ± 0.8 (n = 5)	35.9 ± 0.3 (n = 4)

The WAT significantly influenced the final mean LOE temperatures for both Bluehead and Flannelmouth Sucker larvae (Figures 6.9 and 6.10). Overall, Catostomid larvae exhibited a clear shift in their temperature tolerances as the WAT increased (i.e., display of thermal plasticity). Specifically, with CTMax, Bluehead Sucker larvae tested at a WAT of 13.8ºC had a mean LOE of 28.5ºC ± 3.4. This value increased significantly to a mean LOE of 36.2ºC ± 0.1 when the WAT reached 21.8ºC (Figure 6.9A; $F_{7,76} = 12.58$, $p < 0.05$;). A similar pattern was observed with Flannelmouth Sucker larvae. The CTMax mean LOE was 30.7ºC ± 0.8 at a WAT of13.8ºC, which significantly increased to 35.9ºC ± 0.3 at a WAT of 21.8ºC (Figure 6.9B; $F_{6,64} = 4.69$, $p < 0.05$). In general, intermediate WATs displayed similar CTMax mean LOEs for both species; however, WATs above 18.6ºC were associated with a marked increase in upper thermal tolerance.

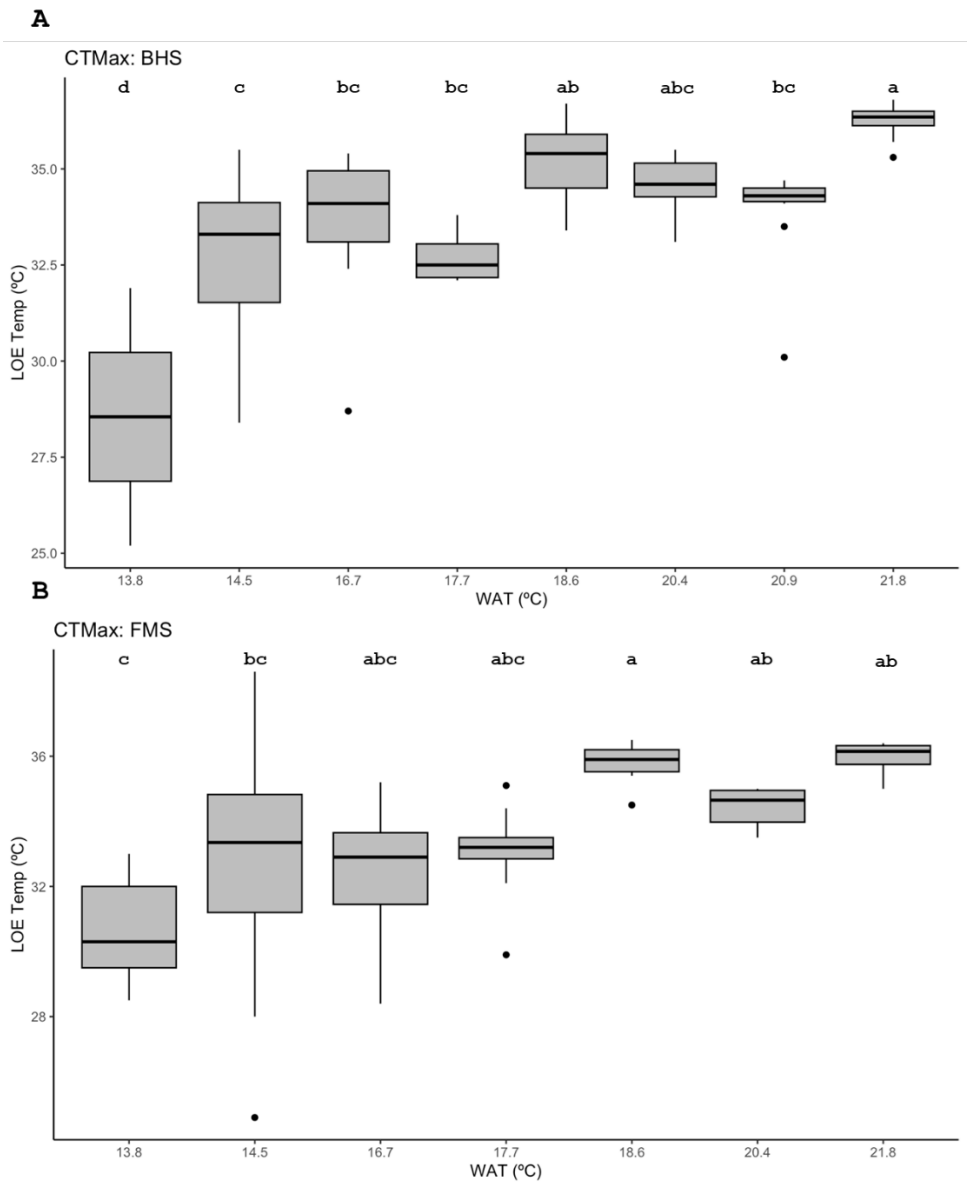

Figure 6.9 Mean loss of equilibrium temperatures (LOE; ºC) reached by Bluehead Sucker (BHS; A) and Flannelmouth Sucker (FMS; B) larvae across a range of weekly average temperatures (WAT) for critical thermal maximum. Interior section of boxplots represents 50% of data, with the darkened horizontal band representing the median. Boxplot outer edges represent the upper quartile (75th percentile; top) and lower quartile (25th percentile; bottom). Points outside of the boxplots signify outliers. Letters above the boxplots are the results of Tukey's Honest Significant Difference of multiple comparison test. Matching letters indicate non-significant relationship between two or more treatments (α>0.05).

The relationship between CTMin and WAT was less pronounced compared to the CTMax relationship (Figure 6.10). Flannelmouth Sucker larvae exhibited differences in mean LOE's across WATs. Larvae acclimated under a WAT of 16.7ºC displayed a mean LOE of 8.9ºC ± 0.6, which was significantly different from 13.8ºC and 14.5ºC (Figure 10B; $F_{6,63} = 2.79$,$p < 0.05$). All other pairwise comparisons for Flannelmouth and Bluehead Sucker larvae did not show significant differences in mean LOE, indicating that CTMin was not as strongly influenced by increasing WAT and CTMax.

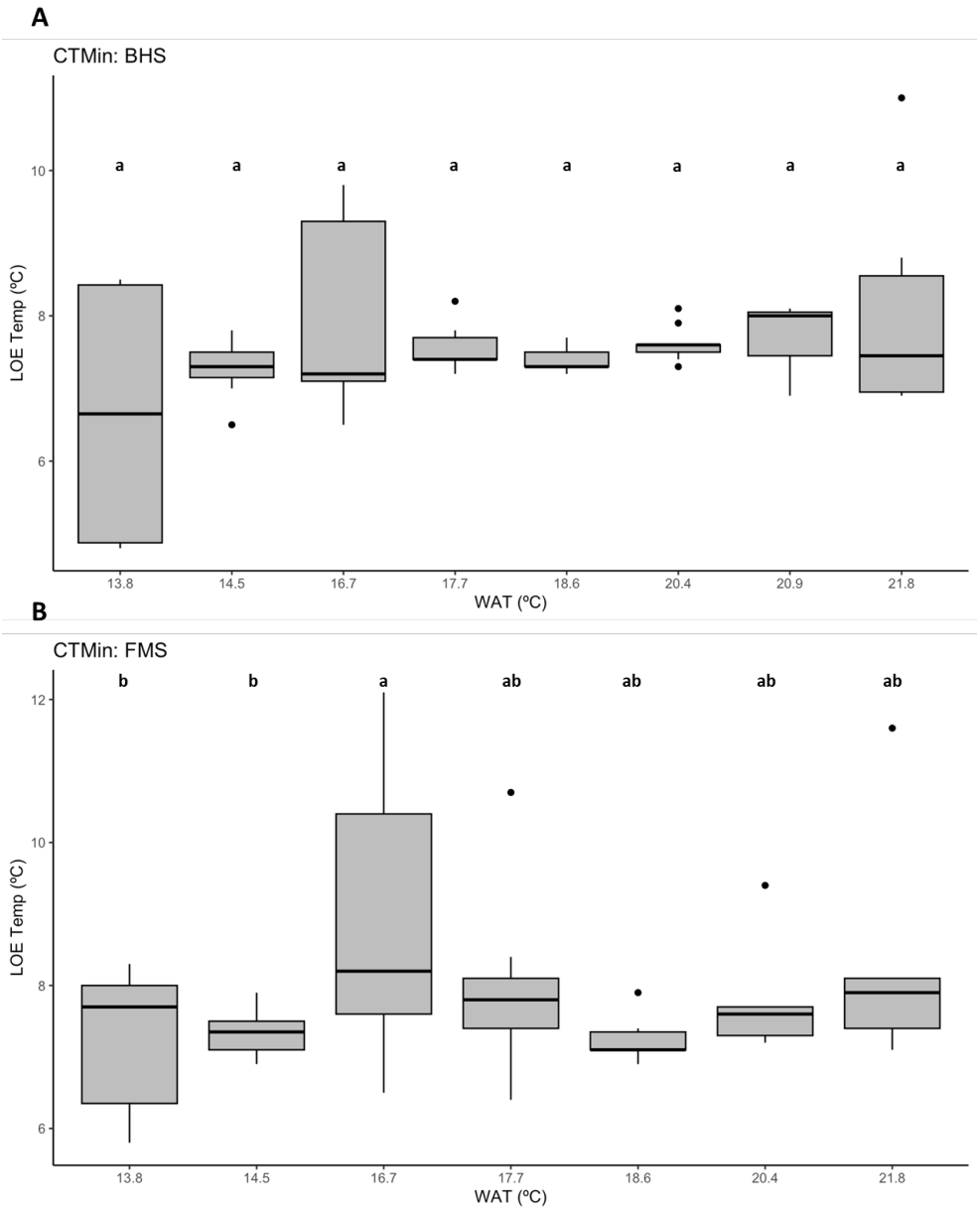

Figure 6.10 Mean loss of equilibrium temperature (LOE; ºC) of Bluehead Sucker (BHS; A) and Flannelmouth Sucker (MS; B) larvae for critical thermal minimum at various weekly average temperatures (WAT). Interior section of boxplots represents 50% of data, with the darkened horizontal band representing the median. Boxplot outer edges represent the upper quartile (75th percentile; top) and lower quartile (25th percentile; bottom). Points outside of the boxplots signify outliers. Letters above the boxplots are the results of Tukey's Honest Significant Difference for multiple comparison test. Matching letters indicate non-significant relationship between two or more treatments.

The simple linear regressions models were used to quantify the positive relationship between WAT and the LOE temperature for both CTMax and CTMin trials. Both CTMax models demonstrated a significant positive linear relationship, indicating that an increase in WAT was directly associated with an increase in upper temperature tolerance (Figure 6.11). For Bluehead Sucker every 1°C increase in WAT, the CTMax LOE was estimated to increase by 0.64ºC (Figure 6.11A; $t_{6,7} = 3.42$, p = 0.01). The WAT was highly predictive explaining 64% of the variance in mean LOE ($R^2 = 0.64$). Flannelmouth Sucker larvae also showed a significant linear relationship, where upper temperature tolerance increased by 0.56ºC for every 1°C increase in WAT (Figure 6.11B; $t_{5,6} = 3.89$, p = 0.01). The Flannelmouth Sucker model explained 75% of the variance in mean CTMax LOE ($R^2 = 0.75$). The ANCOVA comparing the CTMax responses showed no significant species effect ($F_{1,15} = 0.10$,p = 0.76,). This suggests that Bluehead and Flannelmouth Sucker larvae possess similar upper thermal tolerance thresholds and similar rates of response to the WAT.

The relationship between WAT and CTMin was much weaker. Bluehead Sucker CTMin regression model suggested a slight, yet significant, positive association (Figure 6.12A; $t_{6,7} = 2.59$, p = 0.04). A 1°C increase in WAT was associated with only a 0.11°C upward shift in lower temperature tolerance, with WAT explaining 53% of the variation in mean LOE ($R^2 = 0.53$). Flannelmouth Sucker larvae showed no significant linear relationship between WAT and CTMin (Figure 6.12B; p = 0.40). Despite the weak or non-significant results, the CTMin ANCOVA also indicated no significant difference between the lower thermal tolerances of Bluehead and Flannelmouth Sucker larvae ($F_{1,15} = 0.004$, p = 0.95).

6.4 Future Work

As the conclusion of the 2025 field season, the primary data collection phase of the project is complete. We successfully sampled over eight WATs for CTMax and CTMin and three WATs for UILT data, utilizing a custom-designed mobile laboratory suitable for collecting temperature tolerance data in a riverine system. Data analysis is currently underway, yielding the preliminary conclusions reported herein. The future work of this project is structured into two main analytical phases, culminating the final report. The first priority is the finalization of larval identification for all UILT samples. Then we will assess differences in UILT tolerance across species, developmental stages, and water quality parameters. The second phase will focus on generating a comprehensive thermal to visually and quantitatively represent the thermal niche boundaries of the species. These profiles will be used in comparison with CPW's laboratory studies (Riepe et al. 2023; Riepe et al. 2024). This comparison will be critical for understanding the similarities and differences between temperature tolerance between controlled laboratory conditions and the variable environmental of natural systems.

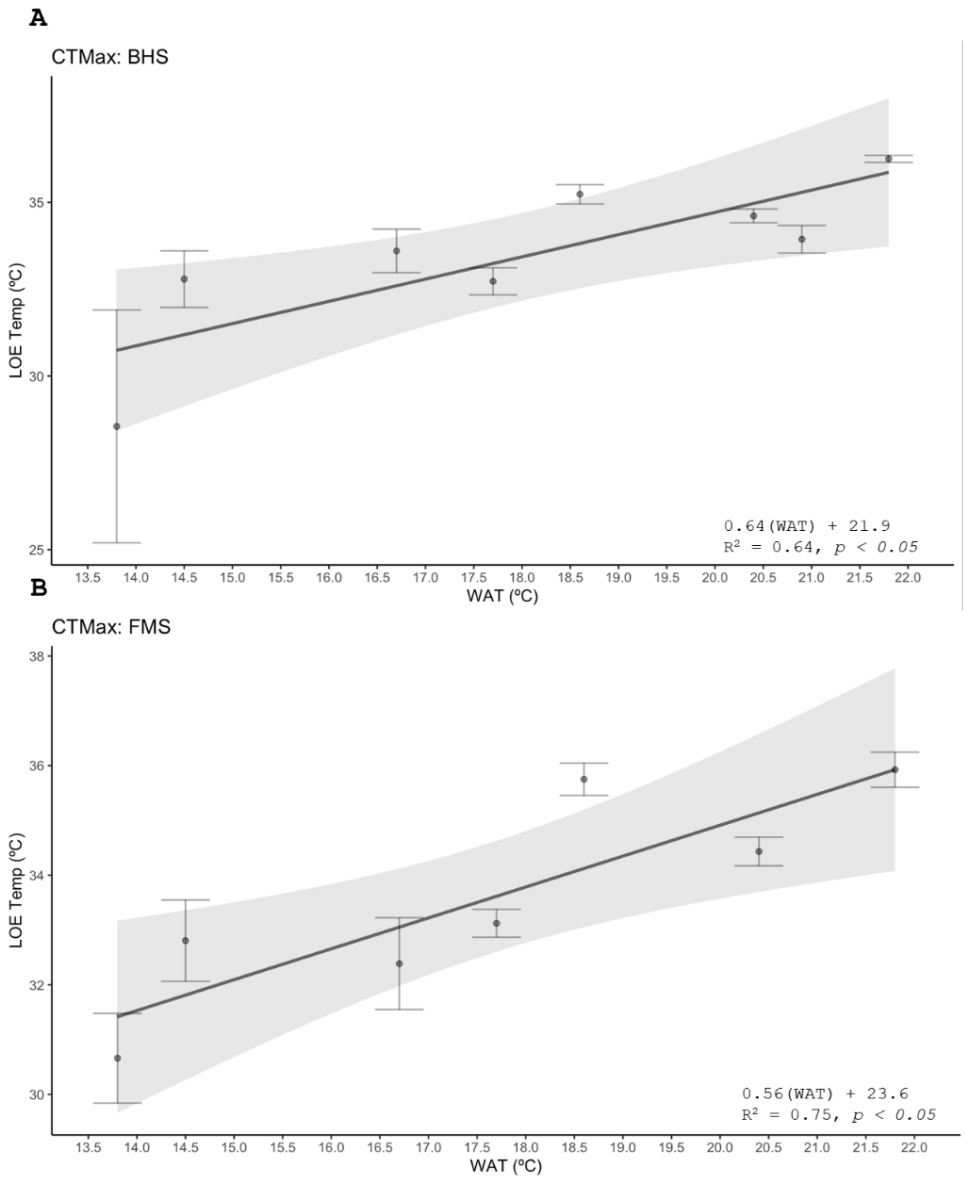

Figure 6.11 Simple linear regressions for Bluehead Sucker (BHS; A) and Flannelmouth Sucker (FMS; A) larvae. Plots illustrate the relationship between weekly average temperature (ºC) and mean loss of equilibrium (ºC) for CTMax trials, with shaded regions representing 95% confidence intervals. Regression equation and model output is overlaid on plot in the bottom right corner.

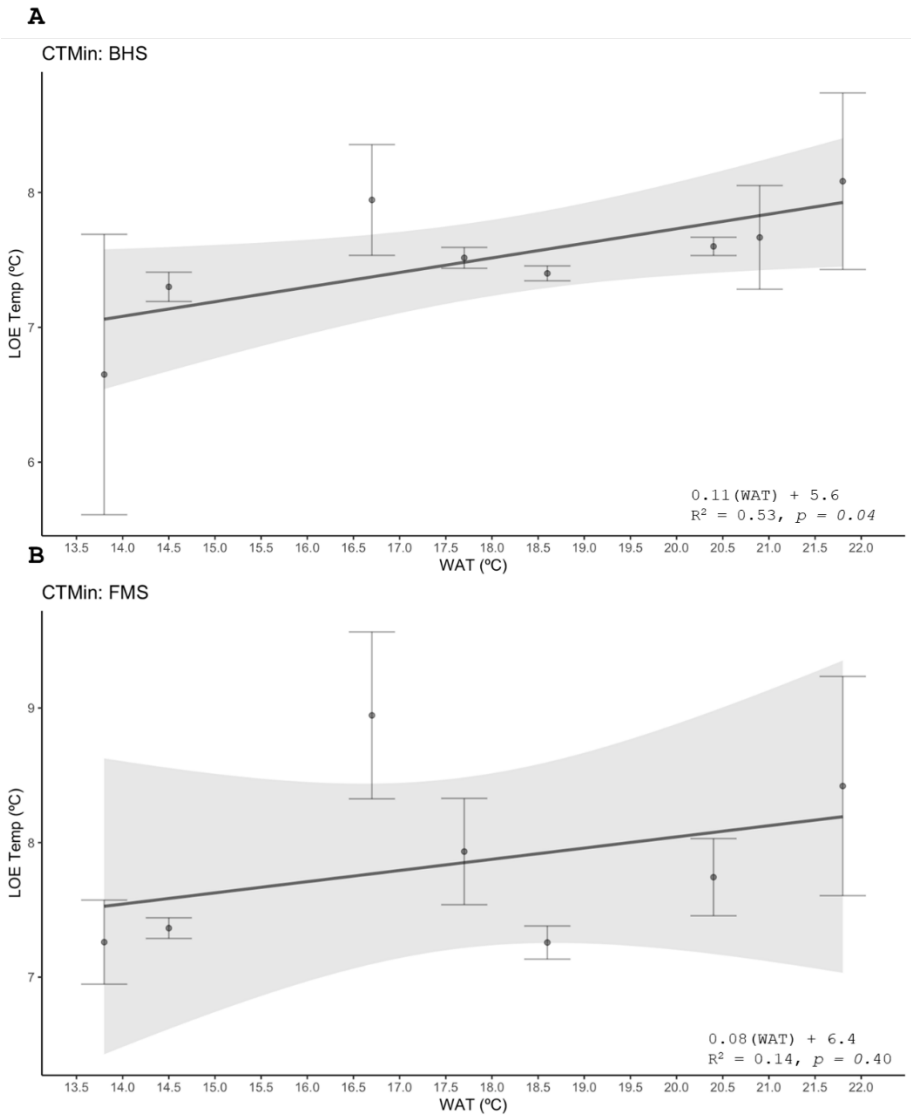

Figure 6.12 Simple linear regressions for Bluehead Sucker (BHS; A) and Flannelmouth Sucker (FMS; A) larvae. Plots illustrate the relationship between weekly average temperature (ºC) and mean loss of equilibrium (ºC) for CTMin trials, with shaded regions representing 95% confidence intervals. Regression equation and model output is overlaid on plot in the bottom right corner.

6.5 References

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