2022

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Recommended Citation

Frakes, James; Andreas, Amanda; Colman, Benjamin P.; and Woods, Aurthur, "COMBINED EFFECTS OF TEMPERATURE AND HEAVY METALS ON THE PERFORMANCE OF THE GIANT SALMONFLY." (2022). *Graduate Student Theses, Dissertations, & Professional Papers*. 11916.
https://scholarworks.umt.edu/etd/11916

COMBINED EFFECTS OF TEMPERATURE AND HEAVY METALS ON THE PERFORMANCE OF THE GIANT SALMONFLY.

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Master of Science, University of Montana, Missoula, MT, 2019

Thesis

Degree: Organismal Ecology: Ecology & Evolution

The University of Montana

Missoula, MT

Official Graduation Date [May 2022]

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Frakes, Jameson, M.S., Spring 2022 *Organismal Biology*

Abstract

In many freshwater ecosystems, communities of aquatic insects are facing the combined stresses of warmer waters due to climate change and increased exposure to heavy metal toxicants. Although each stressor may threaten aquatic insects independently, they also likely interact in important ways to affect insect physiology and performance. Here we investigate this potential interaction using two populations of aquatic nymphs of the giant salmonfly, *Pteronarcys californica*, collected from adjacent rivers in Montana: naïve individuals from Rock Creek, a relatively pristine stream, and individuals from the Upper Clark Fork River, which has a history of heavy metal pollution and higher temperatures. We used a factorial design that exposed nymphs from the two rivers to one of two varying concentrations of metals (copper or lead) in combination with one of two temperatures (12 or 18 °C). We measured survival, growth, and upper critical temperature (CT_{MAX}), as well as individual heavy metal concentration. Nymphs from both rivers exposed to the highest amounts of copper showed reduced survival and growth rates, and their CT_{MAX} were reduced by up to 10 °C. By contrast, lead had little effect on survival, growth, or CT_{MAX} of either population. These results suggest that acute exposure to heavy metals may reduce the ability of aquatic insects to withstand exposure to climate-induced warming.

Keywords

Heavy metals, CT_{MAX}, acclimation, *Pteronarcys californica*, Upper Clark Fork River, climate change, aquatic insect, copper, lead

Introduction

Key threats to aquatic habitats include the multifaceted effects that stem from hotter, more variable climates (IPCC 2020). In Montana, many streams and rivers are warming rapidly compared to similar bodies of water around the globe (Whitlock et al. 2017). This warming poses a challenge to native aquatic taxa, which consist primarily of small ectotherms whose body temperatures are tied closely to their thermal environments (Willmer et al. 2000). Rising temperatures are predicted to exceed the thermal tolerance limits of some native aquatic organisms, which can cause rapid die-offs (Till et al. 2019). Furthermore, sublethal side-effects from changing temperatures may misalign the emergence of aquatic insects with the phenologies of other aspects of their biotic and abiotic environments (Hering et al. 2009, Conti et al. 2014), reduce dispersal distances (Jourdan et al. 2019), decrease the amount of suitable habitat (Taubmann et al. 2011), increase the prevalence of diseases (Marcos-López et al. 2010), and promote the upstream movement of invasive species (Rahel & Olden 2008).

Temperature also interacts with other potential stressors arising from climate change (Birrell et al. 2020) and anthropogenic chemical disturbances, including nutrient loading, agricultural pollutants, and heavy metal toxicity. Although most research studies these stressors independently, to predict the fate of aquatic communities it is critical to assess how warming temperatures interact with other sources of stress (Moe et al. 2013) Understanding interactive effects is especially important in the context of aquatic insects, an abundant group that plays key ecological roles in aquatic ecosystems and often supports higher trophic levels (Canfield et al.

1994, Merritt et al. 2008).

Studies on the combined effects of temperature and toxicants such as heavy metals suggest that they often interact, with warmer temperatures increasing susceptibility to toxicants, and in turn, those toxicants decreasing heat tolerance (Cairns et al. 1975, Sokolova & Lannig 2008, Moe et al. 2013). In aquatic ectotherms, elevated temperatures typically stimulate biological rates, e.g., metabolism, ventilation, feeding, and growth, all processes that can further expose individuals to toxicants and exacerbate their negative effects (Sokolova & Lannig 2008). Respiratory surfaces are often a primary route by which toxicants enter the body (Lannig et al. 2006, Clements 2019), which may directly inhibit the efficiency of gas exchange (Nonnotte et al. 1993). For aquatic insects in particular, respiratory interference from metal exposure would likely reduce their thermal tolerance, given the role of oxygen-transport in setting these limits (Pörtner 2001, Verberk et al. 2016, Frakes et al. 2021).

The Clark Fork River Basin Superfund Complex extends from Butte to Missoula, MT, and is the largest complex of Superfund sites in the United States (Vincent 2012). Historical copper and silver mines near Butte, MT, introduced arsenic, cadmium, copper, lead, and zinc into the upper watershed (Axtmann & Luoma 1991). Subsequent flooding events have washed accumulated metals into the Upper Clark Fork River (further referred to as UCFR) downstream over 300 km (Johns 1995, Stagliano 2020), where metal contamination has been detected across trophic levels from macrophytes to ospreys (Johns 1995, Cain et al. 2004, Langner et al. 2012). Large-scale remediation efforts (e.g., bank sediment removal and the construction of the Warm Springs Settling Ponds) have attempted to restore the UCFR and protect its inhabitants from heavy metal exposure (Vincent 2012); as such, metal levels in the UCFR are now declining. Higher river temperatures stemming from climate change, however, may amplify the effects of the heavy metals that remain (Sokolova & Lannig 2008, Moe et al. 2013). Additionally, increases in flood severity in the future—a consequence of climate change in Montana (Whitlock et al. 2017)—may mobilize toxicants buried in riparian sediment and floodplain soils and produce transient periods of high metal load in the UCFR.

To examine the effects of exposure to the dual stressors of heavy metal contamination (copper or lead) and higher temperatures on aquatic invertebrates, we measured the survival, growth, and critical thermal maxima (CT_{MAX}) of an important stonefly, *Pteronarcys californica* (the giant salmonfly), in a set of lab-based exposure experiments. We predicted that the combination of heavy metal exposure and elevated temperature would interact to reduce salmonfly performance. Warm temperatures may increase the rate of metal exposure and exacerbate its negative effects, or certain metal toxins may decrease salmonflies thermal tolerance, making them more susceptible to high-temperature stress (Sokolova & Lannig 2008). In our experiments, we compared performance and physiology between larval salmonflies collected from the UCFR versus those from a nearby uncontaminated and cooler tributary, Rock Creek, near Clinton, MT (Fig. 1). We envisioned two opposing outcomes: Populations that have experienced stressors (those from the UCFR) may have adapted or acclimated to such stressors, allowing them to outperform populations from cool and uncontaminated places when they are exposed to warm and contaminated water. This would be supported if salmonflies from the UCFR outperformed those from Rock Creek. Alternatively, chronic exposure to stressful conditions like those in the UCFR may negatively impact organismal condition and the capacities of their underlying physiological systems. This would be supported if Rock Creek salmonflies outperformed those from the UCFR during experimental exposure to higher levels of temperature and heavy metals.

Aquatic insects often show strong phenotypic plasticity in their upper thermal limits (Gunderson & Stillman, 2015), likely an important trait to maintain as populations navigate warming climates (Huey et al., 1999). Therefore, we complement the lab experiments with an in-field transplant experiment to assess how the $\mathrm{CT_{MAX}}$ of salmonflies from Rock Creek change when held in mesocosms at three locations along the UCFR during the hottest days of summer (Fig. 1).

Methods

Insect collection

Nymphs of *Pteronarcys californica* that ranged from 0.04 - 1.12 g (supplemental Fig. 1) were collected from Rock Creek, near Clinton, MT (lat, long; 46.688073, -113.662777), and the Clark Fork River, near Phosphate, MT (46.555749, -112.873970), using a kick screen barrier net (1×1 m). Insects were identified on site and placed temporarily into buckets of river water aerated with battery-powered air pumps (Silent Air B10 Aquarium Air Pump, Penn – Plax, Hauppauge, NY). The buckets were transported to the University of Montana on the same day and placed into a temperature-controlled incubator at 12 ºC (I66LLC8; Percival, Perry, IA) with conditioned cottonwood (*Populus*) leaves collected from Rock Creek (food source) for approximately one week. Two identical sets of insect collections occurred on each river, first on July 29th for the copper exposure experiments, and then again on September 9th for the lead exposure experiments.

Experimental design

To determine how salmonflies from Rock Creek and the UCFR respond to combinations of heavy metals and temperature, we used a factorial design that crossed two temperatures (12 ± 3 ºC and 18 ±3 ºC) with three environmentally relevant treatment levels of heavy metals (control, medium, high) for two metals, lead (Pb) and copper (Cu). We exposed 12 insects from both rivers to each combination of temperature and heavy metal concentration.

Prior to experiments, all plastic consumables (aquaria, nylon mesh bags, cable ties, and tubing) were acid washed with 3% trace metal grade nitric acid (A509-500, Fisher Chemical, Waltham, MA) and rinsed with milli-Q water (resistivity 18 MΩ.cm). Each treatment was conducted in a plastic aquarium (60.5 L, Sterilite, Townsend, MA) filled with 50 L of temperature stabilized and chlorine free (degassed) tap water fitted with an aquarium pump (600 Aqua Pump; Rio Plus, Tiapei, Tiawan) and air stone (AP- 8, Danner Manufacturing Inc., China). To mimic diel fluctuations in temperature in the UCFR, temperatures in both treatments were varied by 6 ºC around the mean (3 ºC above during the day and 3 ºC below at night). The pH in each treatment at the end of each experiment was slightly alkaline, ranging from 8.26 – 8.45 (Halo pH meter; Hanna Instruments, Smithfield, RI). The hardness of the tap water used in the experiments ranged from 325 – 393 ppm (samples analyzed by Clearwater Systems, Missoula, MT).

At the start of the experiment, nymphs were briefly blotted dry with a sheet of Kimtech paper and then weighed on a Mettler Toledo balance to the nearest milligram (ME54TE/00, Columbus, OH). Individuals were then placed in custom-sewn nylon mesh bags (12 cm × 15 cm, mesh size

2 mm) with three conditioned cottonwood (*Populus*) leaves, collected from Rock Creek, and then secured with cable ties before being placed into their treatment. Individuals were held for 21 days in the warm treatments and 28 days in the cold treatments.

Copper exposure

At the time of collections (July 29th 2019) the daily mean temperature was ~ 19 ºC in the UCFR (near Garrison, MT) and ~ 16 ºC in Rock Creek (near Clinton, MT) (USGS). Three initial exposure levels were used: control (0 mg/L Cu), a medium (0.5 mg/L Cu), and high (2.0 mg/L Cu). Initial exposure levels were ecologically relevant as they were based on concentrations measured by the USGS in the UCFR (Table 1). Our medium-level copper exposure was 0.5 mg/L Cu, which is approximately the 95th percentile of copper from unfiltered water sampled in the UCFR between the years of 1984 – 2019 (USGS 2021). A high level of 2 mg/L Cu, and a control treatment with no copper added, were also used. A stock solution of copper (1000 mg/L) was prepared using copper sulfate ($CuSO_4$) (Sigma-Aldrich, St. Louis, MO) dissolved in deionized water. Volumes of stock solution were added to each aquarium filled with 50 L of water to achieve the final concentrations listed above. Copper was added to the aquarium four hours prior to the addition of insects, leaves, and bags.

Lead exposure

At the time of collection (September 9th, 2019) the daily mean temperature was ~ 15ºC in the UCFR (near Garrison, MT) and ~ 13ºC in Rock Creek (near Clinton, MT) (USGS). Again, three ecologically relevant levels of lead were chosen: control (0 mg/L Pb), medium (0.065 mg/L Pb), and high (0.5 mg/L Pb; Table 1). A stock solution of lead (1000 mg/L) was prepared using lead acetate ($Pb(C_2H_3O_2)_2$; Sigma-Aldrich, St. Louis, MO) dissolved in deionized water. Volumes of stock solution were added to each aquarium filled with 50 L of water to achieve the final concentrations (Table 1). Lead was added to the aquarium 4 hours prior to the addition of insects, leaves, and bags.

We expected concentrations of metals in the water to peak at the start of the experiment then to decline as the metals bound to organic matter (e.g., leaves, biofilms, and insect bodies) and adsorbed to the plastic surfaces. Thus, insects could take up metals both across the cuticle and via ingested food.

Survival and growth

We assessed survival on the last day of the experiment, and dead individuals were discarded. Growth was calculated directly at the breakdown of experiments by re-weighing each surviving nymph (same as above). We calculate growth rate as (final mass – initial mass) / days in experiment, which we use as our response variable in our analysis.

Critical thermal maxima

Critical thermal maximum (CT_{MAX}) of each surviving nymph was measured two days after the end of the experiment. Nymphs were transferred back into their mesh bags without a food source and held in 15 ºC chlorine-degassed tap water within incubators (I66LLC8; Percival, Perry, IA) before CT_{MAX} began. Measurements of CT_{MAX} were carried out on batches of individuals (≤ 24 nymphs at a time) with two observers monitoring the experiment. Nymphs were held individually in coffee filters that were partially submerged (5 cm) in water (15 ºC) for two minutes before temperature ramping began. Water temperature was then ramped continuously (0.3 ºC/min) using a programmable temperature controller with a ramp/soak function (SK-89810-04; Cole-Parmer, Vernon Hills, IL) and circulated around a large cooler using two aquarium pumps (600 Aqua Pump; Rio Plus, Tiapei, Tiawan). CT_{MAX} was determined as the temperature at which nymphs lost the ability to right themselves after being placed on their backs with forceps after every 1 ºC of change (Lutterschmidt & Hutchison 1997, Frakes et al. 2021).

Metal analyses

Directly following CT_{MAX} experiments, nymphs were gently scrubbed with a small paintbrush and rinsed in deionized water to remove adherent debris. They were then placed into 15 ml conical tubes and dried for 10 days at 60 ºC in a drying oven (VWR 1525; Sheldon Manufacturing Inc., Cornelius, OR), after which they were weighed on a microbalance (± 1 µg; MC5; Sartorius, Göttingen, Germany).

Copper and lead levels in individual nymphs were determined using inductively coupled plasma mass spectrometry (ICP-MS). Insects were digested overnight in 3 ml of trace metal grade nitric acid (Fisher Chemical A509), and the insect-acid mixture was then diluted with 3 ml of deionized water and heated to 98 ºC for two hours. Conical tubes were housed in larger 50 ml tubes and surrounded with aquarium sand to help conduct heat from the hot-block digester (54-well Environmental Express SC 154; Cole Palmer, Vernon Hills, IL). After samples had cooled, each was aliquoted to 1% nitric acid and sent for ICP-MS analysis. ICP-MS was conducted by the Montana Bureau of Mines and Geology’s analytical laboratory at Montana Technological University in Butte, MT (iCAP Qc Quadrupole; Thermo Fisher Scientific, Waltham, MA)

Initial water concentration of copper and lead in each aquarium was analyzed using an ICP-OES (Optima 5300DV; PerkinElmer, Waltham, MA). Water samples were diluted to 10% and acidified to 2% before being analyzed (Table 1). Final concentrations of water were sampled on the last day of the experiment (Table 1). Water samples were acidified to 3% nitric acid and stored in 15 ml conical tubes in a freezer, then directly prior to ICP-MS analysis the water samples were diluted to 1% nitric acid.

Leaf samples were collected at the end of the copper experiment. A random number generator was used to determine which individuals' food would be chosen for ICP-MS analysis. Leaf material was placed in 15 ml conical tubes and dried for 10 days at 60 ºC in a drying oven then weighed on a microbalance (± 1 µg). Dried leaf material was then crushed with an acid-washed glass rod before being digested in 6 ml of 50% nitric acid and heated to 98 ºC for two hours.

River mesocosm experiment

We collected 60 nymphs of *P. californica* from Rock Creek and then held them in mesocosms at four locations (N = 15 per mesocosm) for four weeks (July 15 to August 20, 2020). These included three locations on the UCFR: Bear gulch (most downstream) (46.7121841, -113,3310013), Phosphate (mid-river) (46.5555907, -112.8720703), and Kohrs Bend (most upstream) (46.4977712, -112.7410226), as well as a control site on lower Rock Creek (46.6717222, -113.67169102) (Fig. 1). After the holding period in the mesocosms, nymphs were brought to the University of Montana, and starved at (12 ºC) for 24 hours before CT_{MAX} was conducted (see methods for CT_{MAX} above).

To avoid the potential spread of whirling disease—caused by *Myxobolus cerebralis*, a parasite that interferes with the nervous systems of salmonids and occurs in Rock Creek—we first held the 60 nymphs in chlorine-degassed tap water in an incubator set to 12 ± 3 ºC for 5 days. Water was changed three times during the five-day lab acclimation period. Additionally, on the fifth day, each nymph was cleaned with a small paintbrush and washed in degassed-tap water before being brought to the field. This method was approved by Montana Fish Wildlife and Parks (MTFWP permit number: 27-2020).

Mesocosms were custom built from clear Plexiglas cylinders (20-cm interior diameter) cut into 50-cm lengths. Removable wire-mesh lids (aluminum mesh size = 1 mm) were attached to the ends of the cylinders using two large hose clamps, and the cylinders were cable-tied to cinderblocks and fitted with a 5 m section of rope. At each site, local sediment, cobbles, and leaf litter were added to each mesocosm to provide nymphs with substrate and food. Nymphs were then added and both lids were tightly secured before the mesocosms were submerged into riffles ~70 cm below the surface of the river and oriented parallel to local flows.

Statistical analysis

Growth and $\mathrm{CT}_{\mathrm{MAX}}$ were analyzed using linear models, with metal concentration, incubation temperature, river of origin, and nymphs' initial mass as predictors (R Core Team, 2017). Survival was modeled using generalized linear models, with metal treatment, temperature treatment, river of origin, and initial mass as predictors. The limit of detection on the ICP-MS for lead was 0.0002 mg/L, a value that was higher than the concentration of lead found in digests of wild populations of salmonflies from both the UCFR and Rock Creek. For this reason, one-half of the limit of detection (0.0001mg/L) was assigned to all surviving nymphs, which were then corrected for mass, and used in statistical analysis (see Clarke 1998). The detection limit on the ICP-MS for copper was 0.001 mg/L, a value that was lower than concentrations of copper in salmonflies from both the UCFR and Rock Creek.

Results

Survival

The concentration of copper and river of origin both influenced larval survival, while there were no detectable effects from lead. Copper exposure level influenced nymphal survival ($P < 0.0001$) where over half of nymphs held in 2.0 mg/L Cu died (Fig. 2). Survival was also influenced by river of origin: insects from Rock Creek survived better than did those from the UCFR ($P < 0.001$). In addition, nymphs with smaller starting masses died more frequently ($P < 0.001$). The temperature × copper interaction was not a meaningful predictor of survival in the copper experiment ($P = 0.668$; Fig. 2; Supplemental Table 1). In contrast to copper, survival was high throughout the lead exposure experiments and lead exposure level did not appear to influence survival ($P = 0.368$; Fig. 2), nor did incubation temperature, river of origin, initial mass, or the temperature × lead interaction (Supplemental Table 2).

Growth Rate

Both copper body burden and body size influenced growth rates. Higher body burdens of copper in larvae was associated with reduced growth rates ($P < 0.001$; Fig.3). Growth rate was also lower for salmonflies with larger initial masses (*P* = 0.006; Supplemental Fig. 2). Larger Rock Creek individuals lost more mass than others (*P* <0.001) and Rock Creek salmonflies tended to grow less than UCFR individuals (*P* = 0. 002).

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Lead exposure did not significantly reduce rates of nymphal growth (*P* = 0.196; Fig. 4; Supplemental Table 2). Growth rates were highly variable in the lead experiment, where large nymphs from the UCFR tended to grow more than those from Rock Creek (*P* < 0.0001) (Supplemental Fig. 3). This may result from an increase in food availability in the lead exposed treatments as the acetate bound to $Pb(C_2H_3O_2)_2$ may act as a nutrient promoting the rapid growth of biofilms.

Upper Thermal Tolerance (CT_{MAX})

Body burdens of copper were a robust predictor of CT_{MAX} (*P* < 0.0001), such that the CT_{MAX} of individuals with the highest levels of copper were about 10 ºC lower compared to those with the lowest copper levels (Fig. 5). Temperature also influenced salmonfly CT_{MAX}, where warm-incubated individuals showed slightly higher CT_{MAX} values than cold acclimated individuals (P = 0.018).

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Incubation temperature strongly predicted CT_{MAX} in the lead experiment, such that warm-incubated individuals showed about a 4 ºC increase in CT_{MAX} (Fig. 5). Lead body burden did not influence CT_{MAX} (*P* = 0.655), though initial mass did (*P* = 0.002; Supplemental Table 2).

Water and leaf samples

Observed concentrations of copper and lead in the water were close to that of the expected concentrations, (0, 0.5, and 2.0 mg/L Cu, and 0, 0.065, and 0.5 mg/L Pb; Table 1). Over the duration of the experiment, however, the dissolved concentration of metals in the water decreased by about 98% in the copper experiments and ~99.8% in the lead experiments, as metals were taken up by other solids in the experimental aquaria (Table 1). Levels of copper in the leaves were strongly influenced by exposure levels ($P < 0.0001$) but did not differ between temperature treatments ($P = 0.847$; Supplemental Fig. 4). Leaf samples were not obtained from the lead experiment.

Mesocosm experiment

The values of CT_{MAX} of salmonfly nymphs held in the UCFR were measurably higher than those held in Rock Creek ($P = 0.0003$) but there was no difference detected among sites on the UCFR ($P = 0.4612$; Fig. 6). This increase in CT_{MAX} is mirrored by a ~3 ºC difference in daily mean river temperatures between the UCFR (mean = 18.58 ºC) and Rock Creek (mean = 15.68 ºC) while the mesocosms were deployed (Fig. 6).

Discussion

Many populations of aquatic insects, which comprise 70 to 90% of freshwater biodiversity (Merritt et al. 2008), are being threatened by increasing heavy metal pollution (Zhou et al. 2020) and warming (Whitlock et al. 2017, IPCC 2021, Albert et al. 2021), and these two factors may interact to affect organismal performance (Sokolova & Lannig 2008, Moe et al. 2013). Here we examined how giant salmonfly nymphs (Plecoptera: *Pteronarcys californica*) perform during exposure to copper or lead at two water temperatures. Copper is a biologically essential element, though it can become toxic at high concentrations. By contrast, lead has no biological utility and may be toxic even at low levels (US EPA 2007). Somewhat surprisingly, salmonflies responded very differently after exposure to copper than lead; copper reduced all three performance metrics (survival, growth, and CT_{MAX}) whereas lead had no direct effect on survival, growth or CT_{MAX}.

Heavy metal bioaccumulation may increase in warmer waters, as ectotherm metabolic activity is closely tied to temperature and increased biological rates can further expose organisms to metals thereby exacerbating their negative effects (Lannig et al. 2006, Sokolova & Lannig 2008, Moe et al. 2013). We did not find evidence of higher accumulation of metals in warm-acclimated salmonflies compared to those that were cold-acclimated. This may be due to multiple factors: The aqueous metal concentrations decreased from the start as they bound to other solids in the aquaria, warm-acclimated individuals may have expelled metals during the experiment, or many of the warm-acclimated salmonflies died in the high levels of copper (Fig. 2), potentially due to increased exposure to copper at high temperature.

Another important interaction between heavy metals and temperature is whether the accumulation of heavy metals impairs performance at high temperatures. We found that salmonflies exposed to copper showed lower upper thermal limits. CT_{MAX} values of nymphs that accumulated the highest amounts of copper were about 10 ºC lower than those of control nymphs (Fig. 5). In the future, populations may approach their upper thermal limits more frequently and our results suggest that those from copper polluted habitats may be more at risk to climate change than others.

An important way in which aquatic insects may mitigate the stress of warming is via adaptive plasticity in their upper thermal limits (Huey et al. 1999, Gunderson & Stillman 2015). However, adaptive plasticity, which allows aquatic insects to sense thermal ques from their environment and adjust their physiology to match, may be hindered in metal-polluted habitats. To assess the plasticity of CT_{MAX} in a metal-polluted habitat, we held salmonfly nymphs in mesocosms in three locations along the UCFR and compared their upper thermal limits to those held in Rock Creek (Fig.1). In addition to high metal pollution, the UCFR is about 3ºC warmer in the summer than Rock Creek (Fig. 6). We found that, over a short period of time, salmonflies were seemingly unaffected by the heavy metal pollution in the UCFR and were able to increase their CT_{MAX} (Fig.6). A similar result came from the lead exposure experiments, where lead had no detectable effect on CT_{MAX}, but salmonflies adjusted their CT_{MAX} based on temperature (Fig. 5). Thus, plasticity may partially mitigate the threat of future higher water temperatures to aquatic insects (Gunderson & Stillman 2015). Our copper-exposure experiment, however, provided troubling evidence that some heavy metals can reduce the utility of temperature-induced plasticity and can directly reduce upper thermal limits (Fig. 5).

One outstanding question is the mechanism that causes CT_{MAX} to decrease following copper exposure. Copper may depress CT_{MAX} by binding to and damaging respiratory surfaces, thereby inhibiting oxygen uptake (Spicer &Weber 1991, Nonnotte et al. 1993, Grosell & Wood 2002, Buchwalter & Luoma 2005) In salmonflies, these surfaces are the tracheal gills that protrude from the ventral thorax and ventral abdomen (Supplemental Fig. 5). Such a mechanism of toxicity would be consistent with known mechanistic links between oxygen supply and CT_{MAX} in aquatic insects (Verberk et al. 2016, Pörtner 2017, Frakes et al. 2021). Another possibility is that copper exposure increases oxygen demand (Kapoor & Griffiths 1976), which could lead to mismatches between oxygen supply and demand at lower temperatures (Verberk et al. 2016, Pörtner 2017).

We found mixed evidence regarding population specific responses to combinations of metal and temperature exposure. On the one hand, we found that salmonflies from the UCFR died at higher rates in copper treatments compared to those from Rock Creek (Fig. 2). On the other hand, salmonflies from the UCFR grew faster than Rock Creek salmonflies when exposed to either copper or lead than Rock Creek individuals (Figs. 3 & 4). This result may be partially attributed to the complexity in salmonfly life history: Nymphal salmonflies go through periods of high growth and periods of dormancy (Townsend & Pritchard 1998), which likely occur at different times between rivers with different seasonal characteristics. Therefore, salmonflies from the UCFR may have been entering a phase of rapid growth during the lead exposure experiment, while Rock Creek salmonflies were not.

Summary

Heavy metal pollution in freshwater ecosystems is increasing in many localities globally, with potentially negative consequences for native aquatic taxa. Understanding and predicting these consequences is difficult: threats typically arise from multiple metals simultaneously, which may enter aquatic organisms via multiple pathways, affect them in species-specific ways, and interact in complex ways with abiotic environmental factors. In this experiment, we examined interactions between two common heavy metal toxicants (copper and lead) and temperature. We show that copper reduces the survival, growth, and upper thermal maxima of an important stonefly species in the American West. These results suggest that additional effort should be directed toward understanding interactions among temperature, metals, and other abiotic factors.

Author Contributions

Conceptualization: JIF, ALA, HAW; Methodology: JIF, ALA, BPC, HAW; Investigation: JIF, ALA; Analysis: JIF, ALA, BPC, HAW; writing– original draft: JIF, HAW; Writing- review & editing: ALA, BPC, HAW.

Acknowledgements

This research was supported by the National Science Foundation RII Track-1 Montana Consortium Research for Environmental Water Systems (CREWS, grant number 366300). We thank Reed Traynor, Priya Keller, and Emily Hamant for help collecting insects and running experiments, and October Moynahan and the ECOR Facility for support. We also tank Jackie Timmer from MT Tech University, Butte, MT, for carrying out the ICP-MS metal analysis and David Buchwalter for early discussions on experimental design and methods for measuring metal concentrations.

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Tables

Table 1: Expected and measured concentrations of copper (Cu) and lead (Pb) in spiked experimental water samples. BDL = below detection limit of the ICP-MS (0.001 mg/L Cu & 0.0002 mg/L Pb). Initial measured concentrations were determined by analyzing stock solutions of copper and lead using inductively coupled plasma optical emission spectroscopy (ICP-OES). Final measured concentrations of copper and lead in water samples, taken on the last day of the experiment, were analyzed using ICP-MS.

Water Samples	**[Expected] (mg /L)**	**[Initial Measured] (mg/L)**	**[Final Measured] (mg/L)**
Cu exposure:			
Control	0	0.0015 ± 0.0001	0.0013 ± 0.0002
Medium	0.5	0.50575 ± 0.0633	0.0081 ± 0.0024
High	2	2.023 ± 0.2533	0.0362 ± 0.0038
Pb exposure:			
Control	0	BDL	BDL
Medium	0.065	0.0645 ± 0.00335	BDL
High	0.5	0.4965 ± 0.02575	0.0011 ± 0.0005

Figure captions

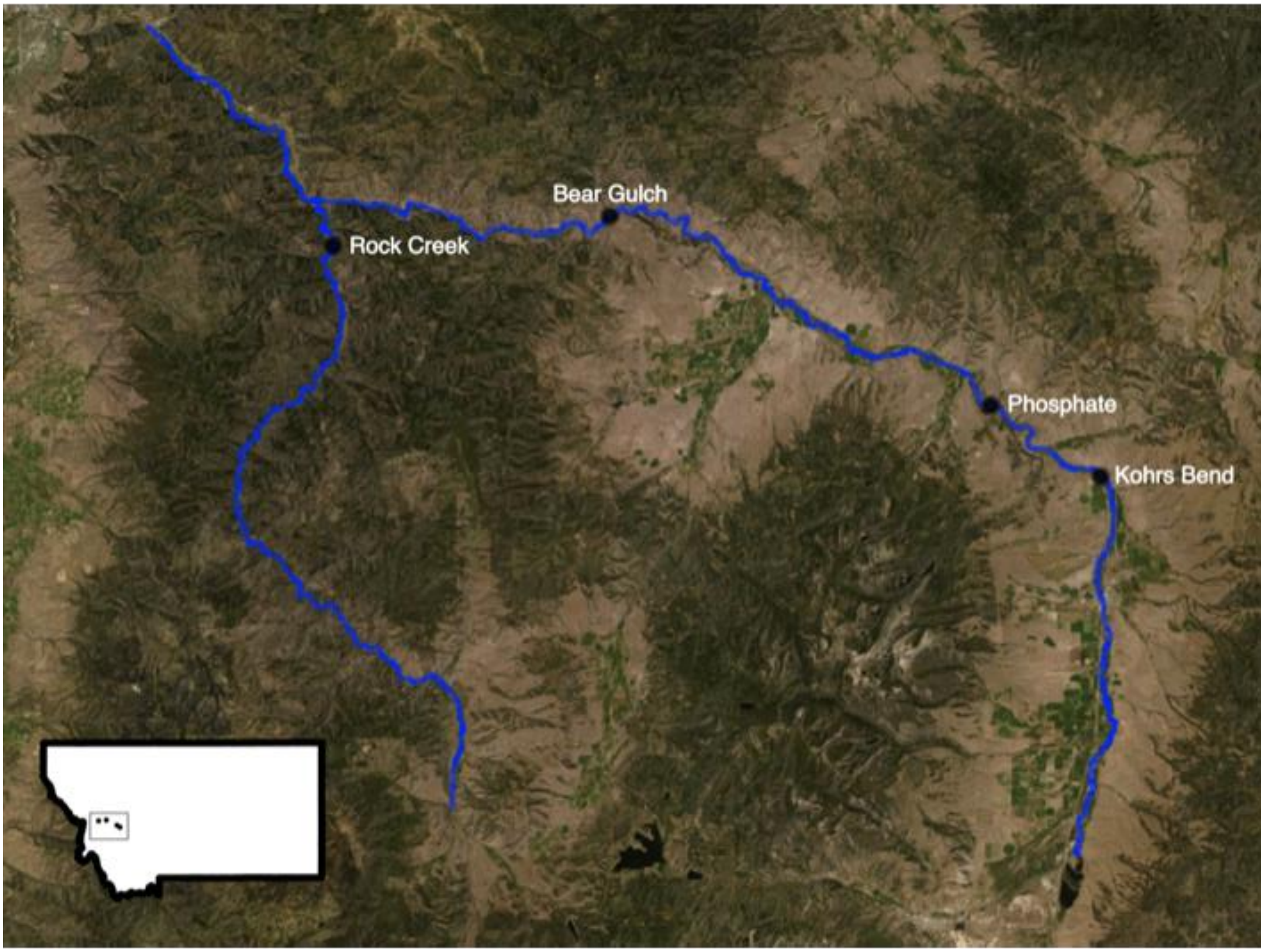

Figure 1: Map of Western Montana, USA, showing the Clark fork River and Rock Creek with salmonfly collection sites (Rock Creek and Phosphate) and mesocosm deployment sites (black dots). *Leaflet Map – source: Esri, I-Cubed, USDA, USGS, AEX, GeoEye, Getmapping, Aerogrid, IGN, IGP, UPR-EGP, and the GIS User Community.*

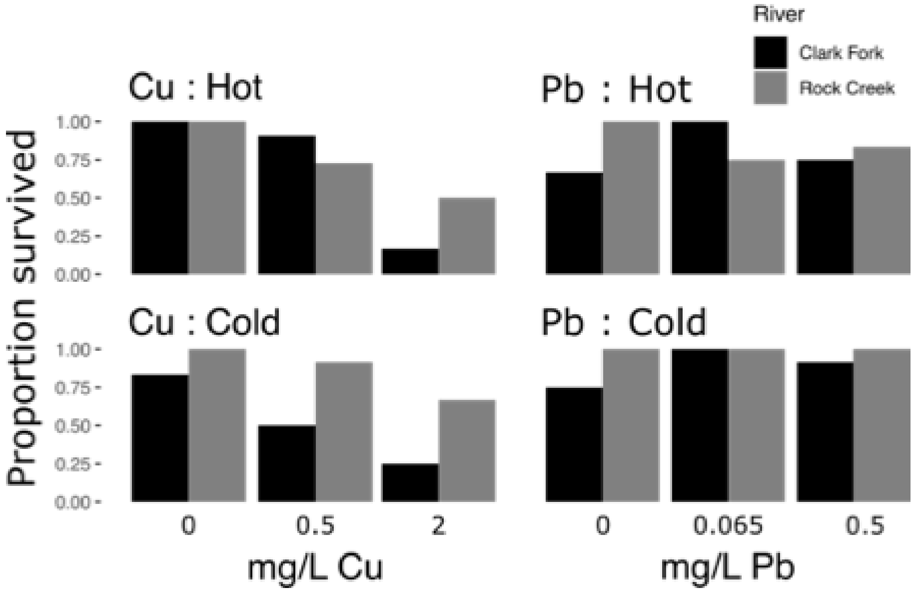

Figure 2: The proportion of salmonfly nymphs that survived in copper (left) and lead (right) exposure experiments and under hot (top) and cold (bottom) incubation regimes.

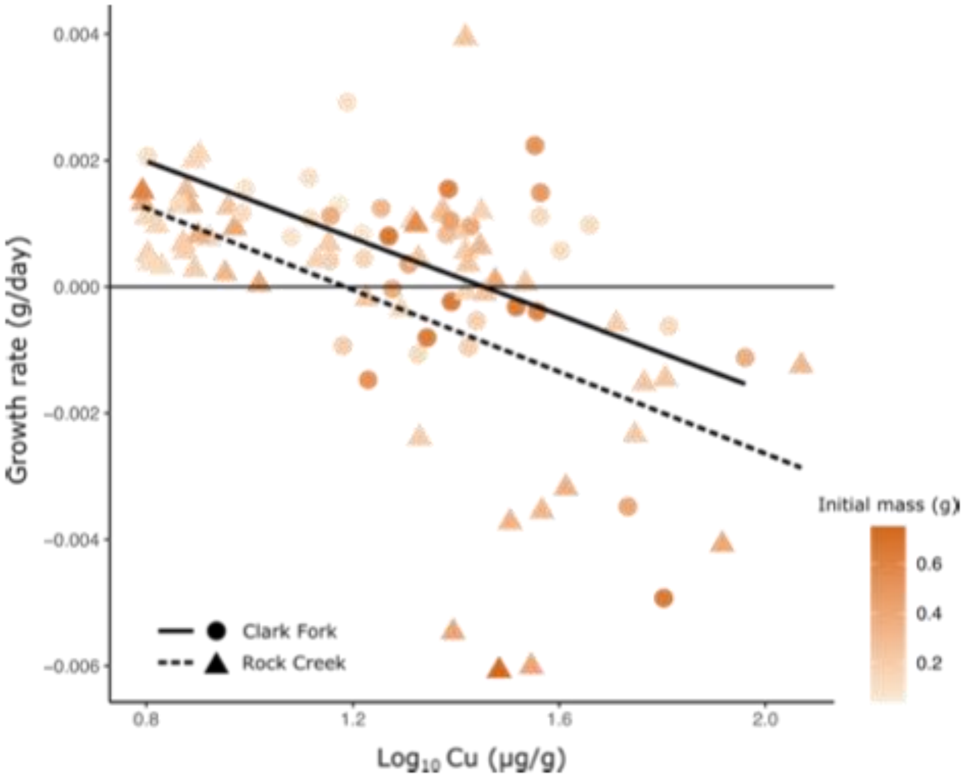

Figure 3: Growth rate as a function of copper concentration (determined by ICP-MS) with initial mass of each nymph indicated by color.

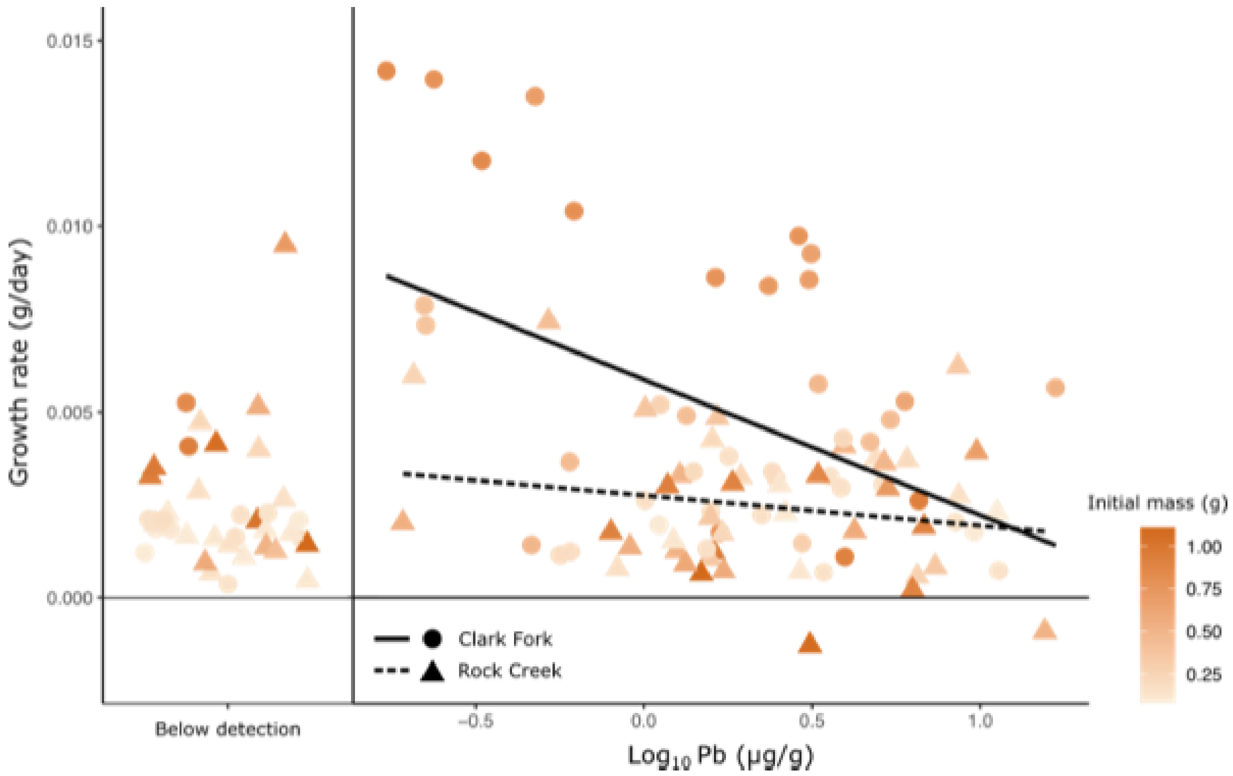

Figure 4: Growth rate as a function of lead concentration with color gradient indicating the nymph's initial mass (g). Nymphs from the control exposure treatment (0 mg/L Pb) had lead concentrations below the detection limit (<0.0002 µg/L Pb) of the ICP-MS and are plotted separately and horizontally jittered.

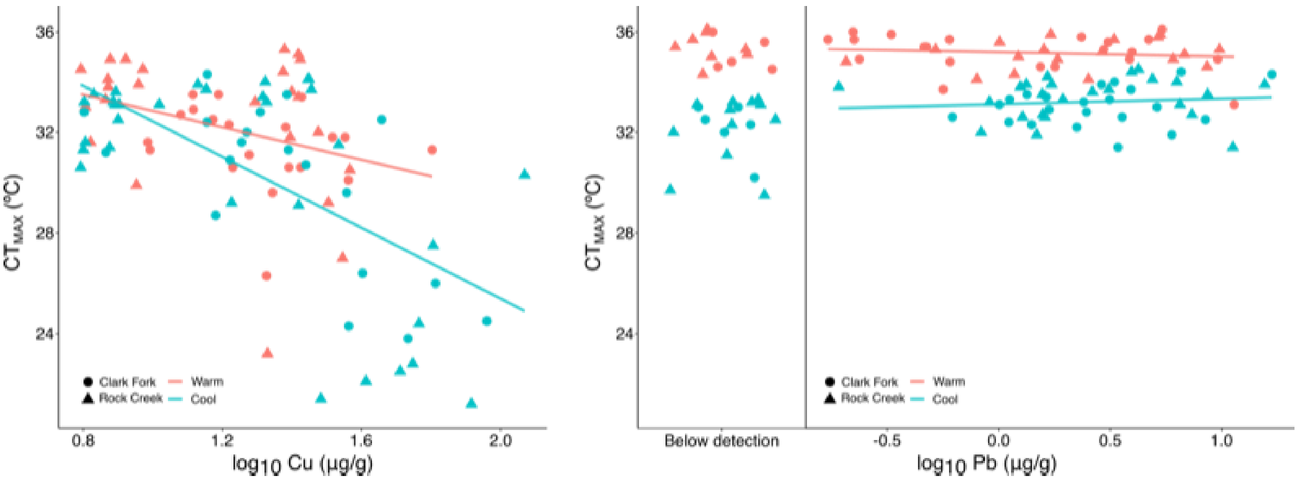

Figure 5: Critical thermal maximum (CT_{MAX}) as a function of the concentrations of copper (left) and lead (right) in individual nymphs. Nymphs below the detection limit of the ICP-MS (<0.0002 µg/L Pb) are plotted separately.

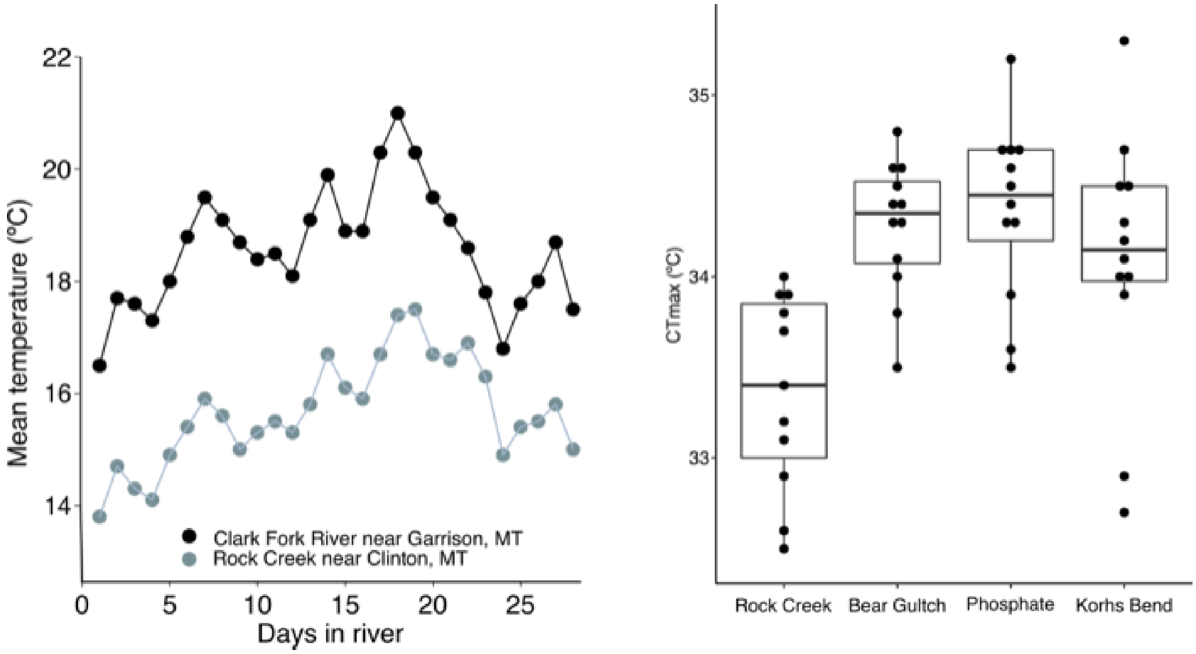

Figure 6: (Left): Daily mean river temperatures from the UCFR near Garrison, MT, and Rock Creek near Clinton, MT, from July 15th – August 12th, 2020 while nymphs were in the mesocosm experiment. Data were obtained from the USGS. (Right) Critical thermal maximum (CT_{MAX}) of salmonfly nymphs collected from Rock Creek that were held in mesocosms at four locations: Rock Creek (control), and three locations on the Upper Clark Fork River (Bear Gulch, Phosphate, and Kohrs Bend, MT).