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Sublethal Toxicity of Copper on Urban Dwelling Damselflies

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SUBLETHAL TOXICITY OF COPPER ON URBAN DWELLING DAMSELFLIES

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LITERATURE REVIEW

Review of sublethal effects of heavy metals on aquatic fauna

Human activity is degrading freshwater ecosystems. We rely on freshwater ecosystems economically, for providing fisheries, clean water, and flood control (Covich et al. 2004). In addition, they are valued for their disproportionate contribution to global biodiversity by supporting 9.5% of Earth's described species despite occupying only 0.8% of the Earth's surface (Dudgeon et al. 2006, Strayer & Dudgeon 2010). Despite their value, human activity is the main source of degradation by water pollution, habitat degradation, invasive species, altered flow regime, climate change, and overfishing (Dudgeon et al. 2006). As a result, freshwater species are more prone to extinction than other ecosystem types (Ricciardi & Rasmussen 1999) including the loss of 32% of amphibian species and 54% of freshwater vertebrate populations (Dudgeon et al. 2006).

As urbanization increases, freshwater ecosystems are more vulnerable to water pollution. Water pollution largely comes from industrial waste runoff that contains elevated nutrients, organic compounds, and metals (Dudgeon et al. 2006). Nutrient pollution contributes to extreme fluctuating dissolved oxygen in water bodies through eutrophication (Davis & Gentley 2000). Other pollutants such as heavy metals and organic compounds are endocrine disruptors, causing cellular stress and sometimes death in aquatic organisms.

Metal pollution is strongly related to urbanization (Xian et al. 2007). The most common metals are cadmium, chromium, copper, lead, manganese, nickel, and zinc

(Beasley & Kneale 2002). Common sources are associated with urban areas and include vehicle brakes and engines, building siding, and dry atmospheric deposition (Davis et al. 2001). The increased amount of impervious surface in urban areas contributes to the high metal loads by diverting rainwater across surfaces rather than allowing it to infiltrate into the ground. As a result, metals are mobilized and accumulated during rain events and subsequently runoff into streams and lakes (Sansalone & Buchberger 1997).

Aquatic organisms accumulate metals when exposed to contaminated water, food, or sediment (Naimo 1995). First they are exposed to it, and then they accumulate metals in their tissue, which can lead to cellular stress and death. Fortunately, many aquatic animals have a variety of defenses to avoid, eliminate, and detoxify pollutants. If the defenses are insufficient the organism dies. If they are sufficient, the associated cost with the defenses often results in behavioral and physiological effects (Callow 1991).

I will review the literature of the sublethal effects in order to explain and predict the individual and community effects of sublethal metal pollution. The review will include macroinvertebrates, fish, and mussels. I will start by explaining the path of toxicity and the defense strategies to reduce toxicity during each step: avoiding contamination, reducing body burden, and detoxification as well as mention the cost for each defense. In part two, I will explain how these costs contribute to physiological effects that reduce individual fitness and can effect community composition.

PART 1: Steps of toxicity / defenses

Avoidance

Avoidance is a behavioral adaptation to contamination. When exposed to a contaminated food, sediment, or water the organism may change its behavior and avoid the sources of contamination by physically avoiding the area, decreasing food consumption, or decreasing burrowing in the sediment. Once removed from the contamination, the organism will resume normal behaviors (Wilding & Maltby 2006). If the organism is unable to fully avoid exposure to contaminants, biogeochemical changes will likely be induced that impact the organism's physiological response.

Mobile organisms avoid concentrated areas by swimming away from high concentration areas and spawning in less contaminated areas. For example, Blunthouse minnows *Pimephales notatus* only spawned in areas less concentrated than 33-77μg/L of copper (Brungs et al.1976). Similarly, *Daphnia longspina* avoided copper contaminated water by staying in chambers with low concentrations. These daphnids exhibited avoidance behavior in chambers at concentrations lower than the LC_{50} **(**Lopes et al. 2004).

Less mobile organisms, such as bivalves, are unable to escape contaminated areas avoid contamination by closing their valve, thereby restricting exposure to the more protective shell. While their valves are normally open to filter food particles and oxygen from the water column, in the presence of metals, they their valves remain closed for a prolonged period of time. The time response for valve closure is within minutes of exposure and decreases at higher metal concentrations (Kramer et al. 1989). Valve

closure in the freshwater mussel *Corbicula fluminea* occurred well below the lethal levels at an EC50 of 4μg Cu/L (Tran et al. 2003).

Mobile organisms also decrease food consumption when food is contaminated. Feeding rate reduction can be attributed to avoidance when feeding rate increases after removal of the contaminant. Both carp *Cyprinus carpio* (De Boeck et al. 1997) and the amphipod *Gammarus pulex* (Wilding & Maltby 2006) reduced food consumption when given food contaminated with Cu and Zn.

Costs:

Costs of avoidance vary between mobile and sedentary organisms. For mobile organisms, escaping to a less contaminated area does not necessarily have any long-term costs unless the movement to other areas depresses foraging. Sedentary animals usually have a greater avoidance cost. Valve closure reduces food consumption (Kramer et al. 1989). Remaining near the top of sediment by reducing burrowing, makes the organism more vulnerable to predators (Bonnard et al. 2009). However, the benefit is that they reduce the amount of metal that is bioaccumulated.

Bioaccumulation

Bioaccumulation occurs when the rate of the metal taken in by the organism exceeds the rate it is eliminated. Metals bioaccumulate in the tissues, exoskeleton, and organs (Rainbow 2007). At chronic exposure to metals, sublethal concentrations can end up accumulating large amounts of metals in the tissue. Metals are endocrine disruptors and cause cellular stress if in a bioavailable form (Rainbow 2007).

Reduce toxic forms of metals

Invertebrates can prevent metals from entering their tissue by secreting mucus. The mucus forms a hard layer on the organism, preventing the metal from accumulating on soft tissue. Increased production of mucus occurs under metal exposure in mussels (Millington & Walker 1983).

A second strategy is to store the metal in a non-toxic form. Vertebrates and invertebrates detoxify the metal by the producing metallothioneins (MT), a protein that binds to metals such as copper, cadmium, zinc, silver, and mercury. MTs expression varies interspecifically and intraspecifically as a function of concentration (Amiard et al. 2006).

Costs:

Both the production of MTs and mucus cost energy. Secretion used approximately 20% of the mollusk energy budget (Callow 1991). Production of MTs on the other hand seemed to be less costly using just 5% of the daphnids energy budget (Barber et al. 1990). These methods of reducing intake of toxic form of metals are energetically costly and likely have fitness consequences (Part II). The reward for these tactics is the reduction of cellular stress.

Oxidative stress

Oxidative stress is the bimolecular damage that occurs when the formation of reactive oxygen species (ROS) exceeds antioxidant defenses. The damage can lead to cancer and death if oxidative stress persists (Halliwell and Gutteridge 2007). Heavy metals induce oxidative stress by the formation of ROS via the Fenton reaction or Haber-

Weiss reaction (Halliwell and Gutteridge 2007). Transition metals can produce a free radical OH when it reacts with H_2O_2. Indicators of oxidative stress include formation of malondialdehyde (MDA) formation and lipid peroxides (Valvanidis et al. 2006).

Oxidative Stress Prevention

Antioxidants and enzymes detoxify ROS. They can also be used as biomarkers of oxidative stress. Indication of ROS formation is the activity of antioxidant enzymes including catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GPx), total glutathione (GSH) and superoxide dismutase (SOD). These biomarkers indicate that there is ROS formation, which leads to oxidative stress if the antioxidant defenses are insufficient. Indicators of oxidative stress include formation of malondialdehyde (MDA) and lipid peroxides (Valvanidis et al. 2006).

A comparison study of two species tolerance to the same concentration of Cu and Zn highlights the importance of the detoxification mechanisms in toxicity. The mussel *Mytilus galloprovincialis* exhibited higher MDA than the oyster *Crassostrea angulata* indicating that the mussels were more sensitive to Cu and Zn contamination and were experiencing oxidative stress. Oysters had higher activity of antioxidant enzymes resulting in lower MDA production. Thus, oysters were better at reducing oxidative damage by producing more enzymes and antioxidants than the mollusk. The oysters even had a greater concentration of Cu and Zn content in their tissues (Funes et al. 2006). Thus, the oyster diverted energy toward antioxidant defenses, which decreased the amount of oxidative stress.

Iron lead to oxidative stress in fish studies. A five-week study on the African catfish *Clarias gariepinus* revealed that an elevated iron diet promoted oxidative stress. This was indicated by depleted vitamin E and an increase in both MDA and lipid peroxidation. The oxidative stress did not result in death but it did reduce growth (Baker et al. 1997). The reduced growth is likely the consequence of the energy cost of the detoxifying enzymatic activity.

Part II – Sublethal Effects

Energy Costs

The energy costs associated with secretion of mucus and detoxification divert energy from reproduction and growth. This metabolic cost is used in scope for growth (SFG) equation, which measures how much energy is available for future growth in terms of both physical growth of the organism and reproduction. SFG is a measurement of how much energy is taken in (food) to how much is used (respiration and excretion). The difference equals the available energy that can be used for reproduction and growth (Widdows and Salkeld 1993).

The metabolic cost of detoxification is revealed by increased respiration rates in the clam *Ruditapes decussatus* when exposed to 10 μg/L of copper. Due to the respiration rates, the measured SFG was depressed, suggesting that growth and reproduction may decrease as well. In the same clam, a higher body burden decreases SFG even after accumulation slows (Sobral and Widdows 1997). Therefore, metals can contribute to energy costs even after the exposure is reduced because the organism is still detoxifying the metal that has already accumulated.

Most studies only measure SFG, but a study on *Daphnia magna* looked at both the growth and reproduction components of SFG. There was a high correlation between the two measurements (Smolders et al. 2005). This supports the metabolic cost theory in that there is likely a physiological effect as a result of the metabolic cost of the stress imposed by pollutants.

Sublethal Endpoints

Reductions in growth rate, reproduction, and activity are sublethal endpoints that result from the energy costs of defenses. As the SFG theory suggests, exposure to pollutants will require maintenance to detoxify and there will be less energy available for growth and reproduction. Furthermore, feeding reduction associated with avoidance behavior can decrease the input of energy, thereby reducing SFG even more noticeably. Thus, due to the allocation of energy toward detoxification and behavioral effects, growth and reproduction decrease during sublethal exposures to metals.

Reproduction is a direct measure of fitness, while growth is strongly related to fitness for many animals including amphibians (Jung & Jagoe 1995), fish (Rosenthal and Alderice 1976), and odonates (Sokolovska et al. 2000). Growth is associated with finding a mate, reproduction, and predation in odonates (Sokolovska et al. 2000). Therefore, exposure to sublethal levels of pollution can still decrease an organism's fitness.

Metals decrease hatchability, reproduction, and growth at sublethal levels in fish (Woltering 1983). The most sensitive response to metals varies by the species which is exhibited in a review of the most sensitive responses. Of the 43 fish species examined, 44% of the species most sensitive response was growth, 11.6% of the species most

sensitive response was hatchability and 30.2% of species was reproduction (Woltering 1983). Not only do species vary in their response, but individuals within a species can have varying responses to metals as well. Two or more physiological responses occurred at the same LOEC (lowest observed effect concentration) for five of the studied species. This suggests that individual responses vary as well. Furthermore, mortality and a physiological effect occurred in 12 species at the same LOEC (Woltering 1983). Thus, fish responses to metals and their associated fitness costs vary both inter- and intraspecifically.

Sublethal effects below LC_{50} are visible during long-term studies. For example, growth reduction did not occur for the Asiatic clam *Corbicula sp*.) until day 20 in a 30 day exposure to 0.5mg/L of zinc (Belanger et al. 1986). Long-term studies on invertebrates also resulted in physiological effects. Sublethal levels of heavy metals decreased growth in the caddisfly larvae *Hydropsyche betteni* (Balch et al. 2000), in the chironomid *Chironomus tetans* (Wentsel et al. 1977), and the daphnid *Daphnia longispina* (Lopes et al. 2006). In shorter 96-h aluminum exposure growth reduction for the amphibian *Hyla cinerea* only occurred at concentrations near the LC_{50}. Exposure to aluminum also induced slower swimming speeds likely due to reduced body size. As a result the tadpoles are more susceptible to predation. (Jung & Jagoe 1995).

The sublethal effect of hypoactivity may also be related to energy costs of detoxificiation. Copper at 1.85ug/L decreased odonate (*Aeshna sitchensis*) and tadpole (*Lithobates sylvaticus*) activity (Hayden et al. 2015). Hypoactivity also decreased in

mussels, fish, and amphibians and has predatory-prey interaction effects (Millington & Walker 1983; Atchison & Sandheinrich 1987; Jung & Jagoe 1995).

Community Effects:

Intraspecific variation can affect the genetic variability of populations. Even at sublethal levels individuals and populations can become less fit due to physiological costs of metals (Callow 1991). Since depressed growth is a common sublethal effect and makes individuals more susceptible to predation, organisms that start off bigger may be favored, as was the case for a population of *Daphnia longispina*. In addition, the variation in body length was less pronounced for historically stressed populations (Lopes et al. 2006). For constantly stressed populations, the genetic diversity can decrease as the resistant or bigger organisms are favored.

Due to the interspecific variation in responses to metal pollution, some species are more vulnerable to metals. Both sublethal and lethal metal pollution can favor the more resistant species and can effect community composition and potentially biodiversity. However, no clear models have demonstrated that these changes to abundance and local extirpation ultimately lead to loss of species over regional scales (Callow 1991).

Conclusion:

Freshwater animals have many defenses preventing lethal toxicity to metals. They can avoid contamination, detoxify with stress proteins, antioxidant enzymes, and metallothioneins. However, these defenses can depress the energy for reproduction, growth, and maintenance. Thus metals can still affect the organism's fitness even at sublethal levels. As literature expands to study the biomarkers of enzymatic activity,

tissue accumulation, scope for energy, and behavioral and physiological endpoints, sublethal effects are being more comprehensively understood. The inter- and intraspecific variation in organismic response to metal contamination indicate that sublethal effects could significantly influence community composition especially in human-dominated ecosystems.

Sublethal toxicity in Urban-Dwelling Damselflies

INTRODUCTION

Freshwater ecosystems contribute disproportionately to global biodiversity by supporting 9.5% of Earth's described species despite occupying only 0.8% of the Earth's surface (Dudgeon et al. 2006, Strayer & Dudgeon 2010). Furthermore, freshwater species are more prone to extinction as compared to other ecosystem types (Ricciardi & Rasmussen, 1999). These enhanced extinction rates result primarily from human activities in the upstream watersheds that concentrate pollutants and increase the speed at which rainfall is conveyed off the landscape (Paul & Meyer 2001).

Watershed urbanization in particular leads to predictable shifts in the hydrologic, thermal, and chemical regimes of receiving waters that often extirpate sensitive aquatic organisms (Paul and Meyer 2001, Allan 2004, Walsh et al. 2005). On the other hand, the creation of aquatic ecosystems in urban environments (e.g. stormwater ponds, drinking water reservoirs, or other water features) also provides habitat for freshwater organisms that can promote freshwater biodiversity across urban landscapes (Goertzen & Suhling, 2012; Hassel, 2014). However, even when organisms can survive in these newly constructed urban waterbodies, the pollutants that concentrate in the water and sediment frequently accumulate in the tissues of the resident organisms and interfere with chemical processes (Stephanson et al. 2012; Hassel 2014). At low levels, organisms can detoxify the pollutants, but the maintenance requires an energy cost. Thus, while biodiversity might be maintained at the landscape scale, ecosystem functionality at the local scale may

be impaired due to sublethal toxic effects of concentrated pollutants. (e.g. lower growth rates and reproductive rates).

In urban areas, increased amounts of impervious surface divert rain water across surfaces rather than allowing it to infiltrate into the ground. As a result, rain water from urban storm events flows across roads and parking lots, mobilizing and accumulating heavy metals, nutrients, and other pollutants in the process. After the first flush of a rain event, many metals (e.g. cadmium, copper, and zinc) exceed permissible concentrations allowed by EPA standards (Sansalone 1997). For example, copper (Cu), a common discharged priority pollutant (Beasley & Kneale 2002), was 2-18 times greater than the EPA standards after rain events (Sansalone 1997). Vehicle brakes, building siding, and dry atmospheric deposition are the greatest contributors to Cu pollution (Davis et al., 2001). Urban ponds are often explicitly constructed to sequester these pollutants so that they are not allowed to reach receiving streams (Wu et al., 1996; Hassel, 2014). After storm events, metal sequestration can increase 2-10 times in urban ponds (Wu et al., 1996).

EPA sets a water quality criteria (WQC) for any pollutant to protect 95% of freshwater genera from *lethal losses* (i.e. death). For example, for lethal losses due to copper, the final acute value of 4.67 μg/L was derived by evaluating 29 toxicity tests that experimentally measure the critical lethal accumulation (LC_{50}) for a total of 6 invertebrates species and 10 fish species (EPA). This suite of organisms represents a minute fraction of the total number of freshwater organisms that often live freshwaters. Furthermore, EPA's focus on toxicity tests (i.e. lethality) to establish the WQC fails to

account for sublethal effects. For example, many metals that accumulate in urban ponds have been shown to depress growth rates, impair enzyme activity, and lower reproductive rates (Beasley 2002). Pollutants also cause behavioral changes that influence predator-prey interactions (Hayden et al., 2014). Finally, EPA's standard methods does not test whether diet is a significant route for accumulation of toxic substances in organisms, since food is not routinely administered in tests and if it is, it is required to be below standard metal concentrations (EPA 2002). Because of these shortcomings, EPA's WQC for most pollutants do not necessarily measure the full range of processes important for maintaining ecosystem integrity.

One order of aquatic organisms that the EPA does not typically include in its development of WQC is Odonata, the dragonflies (suborder Anisoptera) and damselflies (suborder Zygoptera). Apart from their aesthetic value, odonates are an important group to urban ponds as a top predator of invertebrates and prey for fish and amphibians. (Simaika & Samways 2008). Even though the development of urban ponds has been shown to improve odonate biodiversity (Goertzen & Suhling 2013), lethal and sublethal effects of urban pollutants on odonates have received much less attention.

Odonates typically do not experience lethal losses until copper concentrations reached several orders of magnitude above the WQC for copper (0.145 µM) (Tollett et al. 2009). However, there have been no studies on the physiological endpoints from copper on odonates and the few metal studies have been combined with pollutants or high pH. For instance, aluminum and pH decreased respiratory activity for *Libellula julia*, but the independent effect of aluminum was not tested (Rockwood et al. 1990). Another odonate

study sampled several different polluted ponds to find out which pollutants were associated with growth. Growth was correlated mostly to pesticides, rather than metals. However, the ponds had a higher concentration of pesticides than metals (Van Praet et al. 2014). Both sublethal endpoints of decreased respiration and growth negatively affect fitness (Sokolovska et al. 2000). By understanding the isolated effects of metals on these endpoints we can better understand the impacts metals have on odonate fitness.

The magnitude of sublethal effects often depends on the route of exposure. The route of exposure effects the location of metal accumulation (Hare 1992). For most insects, aqueous metal exposure accumulates on the exoskeleton, while dietary exposure accumulates internally (Hare 1992). This location may contribute to whether the metal contributes to oxidative stress and lethal or sublethal effects. The combined dietary and aqueous exposure can also interact to create a greater sublethal effect. For example, the combined exposure to zinc in leaves and in water decreased feeding rate of *Gammarus pulex* to a greater magnitude than the addition of their isolated effects (Wilding & Maltby 2006). Furthermore, in a real pond, organisms upon which odonates prey would also have accumulated metal concentrations that could potentially biomagnify in the predator (Timmermans et al. 1989). To my knowledge, no studies have assessed the effect of sublethal concentrations or the interaction of water and food concentration of copper in odonate populations.

Thus, in this study, I asked: *"Do sublethal doses of copper in the ambient water and food source cause physiological changes that alter growth rate in odonates?"* To answer this question, I (a) determine whether sublethal concentrations of copper, both in

ambient water and in prey, decrease the growth rate of the damselfly; (b) assess whether there is a synergistic effect of the combination of elevated copper in both prey and ambient water on odonate growth rate; and (c) assess the degree to which copper bioaccumulates in the bodies of resident odonates. I hypothesized that copper in the surrounding water and in prey will decrease the growth rate of odonate larva synergistically such that growth rates will be more depressed than would be predicted from adding the effects of ambient water and food source alone.

MATERIALS AND METHODS

Pilot study

We conducted a pilot study to assess the sublethal copper concentration range for *Ischnura sp.* (Odonata: Coenagrionidae) larvae and their prey, *Daphnia magna*. This range was then used to choose the concentration for growing *D. magna* to be used as the contaminated food treatment in the main study and to pick the sublethal copper concentration for the aqueous exposure treatment. We also analyzed Cu body burden in damselfly tissue using atomic absorption spectrometry (AAS) to determine the copper concentration that was both sublethal and that would cause bioaccumulation. This concentration was used as the aqueous copper exposure treatment. Damselflies were exposed to 4 copper concentrations that ranged from 2.5μg Cu /L to 2500μg/L for seven days. We tested 6 copper concentrations (1.5μg- 250μg/L) on *D. magna* during a 96-h exposure.

15 individual damselflies were collected on December 11, 2015 at Lowell Ponds State Wildlife Area, Denver CO (39°47'36" N, 105.2'5.8"W) in the littoral zone. Larvae were collected using benthic grab samples with a 500-μm D-net. Larvae were then transferred to the lab and remained in the collected pond water with an aerator for 24 hours to acclimate to room temperature**.**

After a 24-h acclimation, larvae were randomly assigned to treatment. Treatments consisted of five concentrations of copper: 0, 2.5, 25, 250, and 2500 μg/L. We used a log scale range of copper concentrations that was one order of magnitude greater than

reported lethal concentration for odonates *Pachydiplax longipennis* (Tollett et al. 2009) and one order of magnitude less than the LC_{50} for *D. magna* (EPA 2007).

There were three larvae per treatment. Each larva was contained in its own 16-oz. plastic cup that contained 150 mL of the respective copper concentration added to reconstituted hard water (FETAX, Table 1). The concentrations were made from a 5mg/L Cu stock solution prepared by dissolving 0.00197 g of copper sulfate pentahydrate $CuSO_4 \cdot 5\ H_2O$ into 100 mL of dechlorinated water. During the experiment, larvae were fed three *D. magna* every other day and survival was determined by gentle prodding.

Table 1: FETAX solution (ASTM 2000; reconstituted hard water)
All salts were weighed and dissolved in 1 L of MiliQ water. (pH ~ 7.6-7.9)
Hardness = 84.4 mg/L of $CaCO_3$

Sodium chloride, NaCl	625 mg
Sodium bicarbonate, $NaHCO_3$	96 mg
Potassium chloride, KCl	30 mg
Calcium chloride, CaCl2	15 mg
Calcium sulfate CaSO4,	15 mg
Magnesium sulfate, MgSO4	75 mg

After one week of incubation, we froze the larvae in individual glass vials for later tissue analysis. After 1 month, we rinsed with MilliQ water and blotted with a KimWipe ® . The larvae were then weighed on an analytical balance. We first tared the glass beaker with a test tube then added the larva to the test-tube. In this pilot study, we converted wet weights to dry weights using reported odonate average dry: wet ratio of 10% (Tollett et al. 2009). Then we measured the mean concentration of copper from tissue digestates using AAS (EPA, 1994).

During the pilot study, we assessed the lethal copper concentrations for the odonate prey, *D. magna*. The *D. magna* were exposed to seven copper concentrations: 0, 1.25, 2.5, 12.5, 25, 125, and 250μg/L. The same 5 mg Cu/L stock solution was used to make the six copper solutions. Five *D. magna* were placed into a test-tube that contained 25 mL of the respective Cu-solution. During the 72-hour exposure, the *D. magna* were not fed and survival was determined every 24 hours.

From the results of the pilot study we choose to expose the damselflies and their prey, *D. magna*, to different concentrations, due to their different tolerances. We used the highest sublethal concentration for the damselfly, 2500 μg/L and chose 15 μg Cu/L for the *D. magna* exposure because it was just under the concentration that killed all the daphnids.

Experimental Design

A total of 28 larvae were used in the subsequent 10-day experiment after the pilot study. To test for the possible interaction of Cu-contaminated food and ambient water, we used a 2x2 factorial design, with 4 component groups and 7 larvae in each group. Half of the 28 larvae were exposed to 2500μg/Cu water and half in 0 μg/L. In each water treatment, 7 were fed *D. magna* grown in 15 μg/L Cu and the other 7 were fed the control *D. magna* (Figure 1).

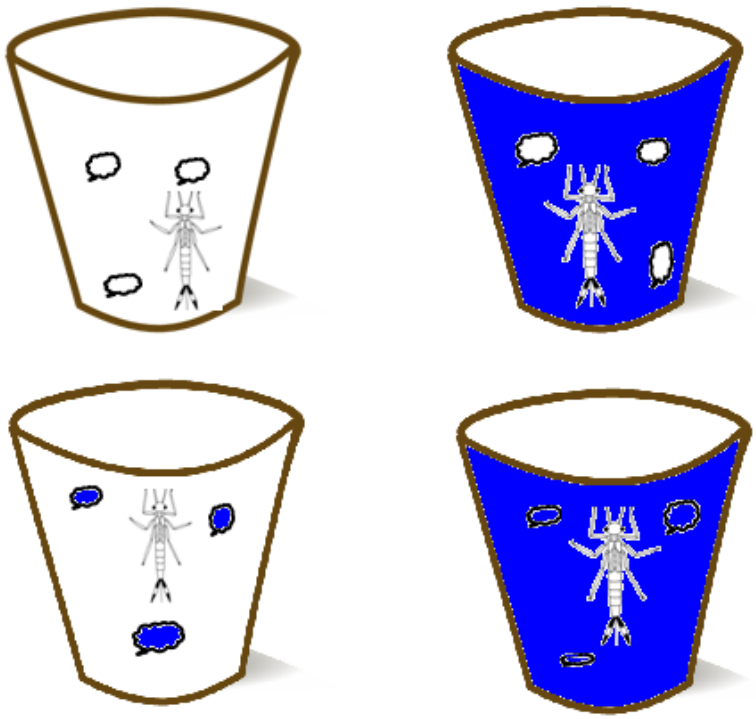

Figure 1: Diagram of factorial design. There are 7 larvae per group, for a total of 28 larvae. Two groups are exposed to copper water (2.5mg/L) and two in control water. One group in the control water and one group in the copper water are fed *D. magna* grown in 15µg Cu/L. The other two groups are fed the *D. magna* grown in control water.

Each culture was maintained in a polyethylene container that contained four L of reconstituted hard water (FETAX solution, Table 1), one dosed with 15 µg/L Cu and the other left undosed. The *D. magna* were grown in their respective concentrations 10 days prior to the experiment where they remained throughout the experiment. Each day, the cultures were fed 5 mL of an algae solution made by grinding an equal volume mixture of algae, yeast, and white flour with a mortal and pestle. 5 g of the composite food mixture was dissolved in 1 L of MilliQ water and stirred for 24 hours with a stirring plate. The resulting solution was then covered with Parafilm and cooled in a refrigerator for an

additional 24 hours to settle out particles. The decantate from the settled solution was used to feed the *D. magna* cultures.

Damselfly Incubation

60 damselfly larvae were collected at Lowell Ponds, in Denver CO on February 18, 2016. Larvae were collected using benthic grab samples with a 500-µm D-net. Larvae were then transferred to the lab and remained in the collected pond water for 24 hours to acclimate to room temperature**.** Larvae around 15 mm of the most common species, *Ischnura sp.*, were separated to a different container and used in the experiment.

Initial weight of each larva was taken before commencing the experiment. However, while the excess water was removed from the weighing boat, the body was not blotted contributing to an overestimation of initial weights. Consequently, we used the wet weight on day 2 as the initial weight of the larvae. Each weighed larva was randomly placed in pre-numbered 16-oz. plastic cups that each contained 150 mL FETAX solution. A 1 g/L Cu stock solution was prepared by dissolving 0.197 g of copper sulfate pentahydrate $CuSO_4\,5H_2O$ into 50mL of MilliQ water. 375µL of stock solution was pipetted into 150 mL of FETAX solution to make the 2500 µg/L Cu solution in the treatment cups. At the commencement of the experiment, each larva was provided with three *D. magna* individuals for food.

An additional 10 damselfly larvae from the benthic grab were used to estimate a log-log regression that predicts dry weight from wet weight. Wet weight was measured for all larvae by placing them on a weighing boat, blotting them dry with a KimWipe ®,

and weighing them on an analytical balance. Dry weight was measured for the same larvae after drying them for 48 hours @ 65 ºC in a drying oven.

Physiological Endpoints

During the 10-day incubation, molting and survival of damselflies were checked daily. Survival was determined by the larva exhibiting motion in response to prodding. Dead *D. magna* were removed from container and dead damselflies were immediately weighed and then frozen. Every other day, larvae were weighed and fed with additional *D. magna* so that the total live *D. magna* was three in the container. Starting on day 7, the number of supplemented D. magna was recorded on the additional feedings. On day 5 the water for all treatments was changed. At the end of the experiment, we took final wet weights and then froze all larvae. We analyzed growth rate by the change in dry weight over time with the equation:

$$\text{Growth} = \ln(\text{final dry weight}/\ \text{initial dry weight})/\text{number of days}$$

Cu body burden

To assess Cu body burden we digested the tissue and then performed AAS. The frozen larvae were thawed and rinsed with Milli-Q water and then transferred to a 23 mL acid-washed test tube. To ensure that the tissue would be completely digested, we pressed each body to the bottom of the test tube with a stirring rod, which was rinsed 3 times with MilliQ water between each larva. 100 µL of the 1g/L Cu stock solution (as $CuSO_4 \cdot 5H_2O$) was pipetted into three test tubes to act as a positive control and three test tubes without any Cu acted as negative controls. Ten *D. magna* grown in the 15 µg/L Cu solution were added to a test tube to examine the concentration of the prey. We pipetted 2 mL of trace

metal grade nitric acid to each tube, capped them and heated them for 12 hours in a constant 75 degree Celsius water bath. The water level in the bath was 2 cm higher than the acid level in test tube to ensure sufficient reflux. After 12 hours, we quantitatively transferred each of the digestates to its own acid-washed 50-mL centrifuge tube and diluted to 15 mL total volume with MilliQ water. We then quantitated copper in the resulting solutions using flame AAS on a Perkin-Elmer AAnalyst 100 instrument (detection limit 0.0770 μg Cu/g dry wt.). The total body burden of copper in each individual was determined by: Concentration* 0.015L/(dry weight)

Statistical Analysis

Statistical analysis of responses (feeding rate, growth rate, molting, body burden, and mortality) involved linear models: analysis of variance (ANOVA), generalized linear models, and multiple linear regression as appropriate to compare the effects of copper-treated water and copper-treated food and their interactions. All models were run in the statistical package R version (R Core Team 2014).

RESULTS

Pilot Study

All damselflies in all treatments survived the 7-day exposure. The copper concentration in the tissue generally increased as the exposure concentration increased, but the trend was not significant (p=0.18, Figure 2). *Daphnia magna* were less tolerant than the damselflies. After 24 hours, individual *D. magna* had died in all treatments. At 48 hours, all five *D. magna* were dead in the highest copper-concentrations: 25, 150, and 250µg/L (Table 2).

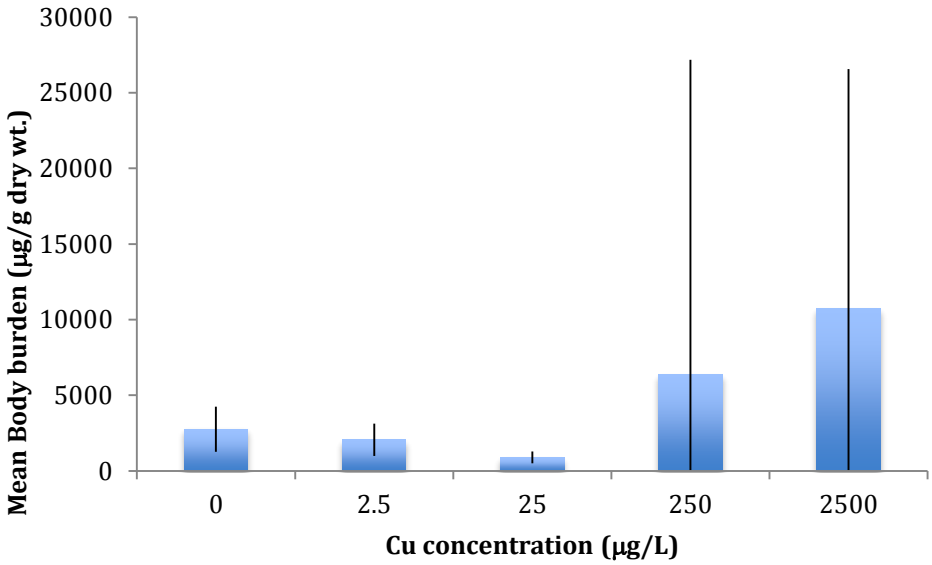

Figure 2: Average larval body burden (µg Cu/g dry wt) +/- SD with 5 copper concentration exposures (n=3 per treatment). Body burdens varied greatly within each sample.

Table 2: Percent survival of *D. magna* exposed to a series of Cu concentrations.

Cu conc. (μg/L)	24-h	48-h	72-h
250	20	0	0
125	0	0	0
25	40	0	0
12.5	60	20	0
2.5	20	0	0
1.5	80	40	0
0	100	40	0

Main Experiment

Interaction between Copper Food and Copper Water Treatments

I compared two-way ANOVA models with and without an interaction term and found that adding an interaction term did not significantly improve model fit for the effects: molting (deviance reduction = 0.30718); mortality (deviance reduction =1.4336e-9); growth (p=0.97); feeding rate p=0.62) body burden (p=0.4). Therefore, the results described below are for the additive two-way ANOVA model without the interaction term.

Initial Weight

Initial dry weight was not significantly different between treatment groups (Figure 4, p=0.104). Day 2 wet weights were used as the initial weights due to inaccurate weighing on first day. Average initial dry weight was 1.7 mg. The larvae wet weights are related to dry weights with the equation:

$$\ln(\text{dry weight}) = 0.7855*\ln(\text{wet-weight}) - 1.5739 \quad (p<0.001, \text{Figure 3}).$$

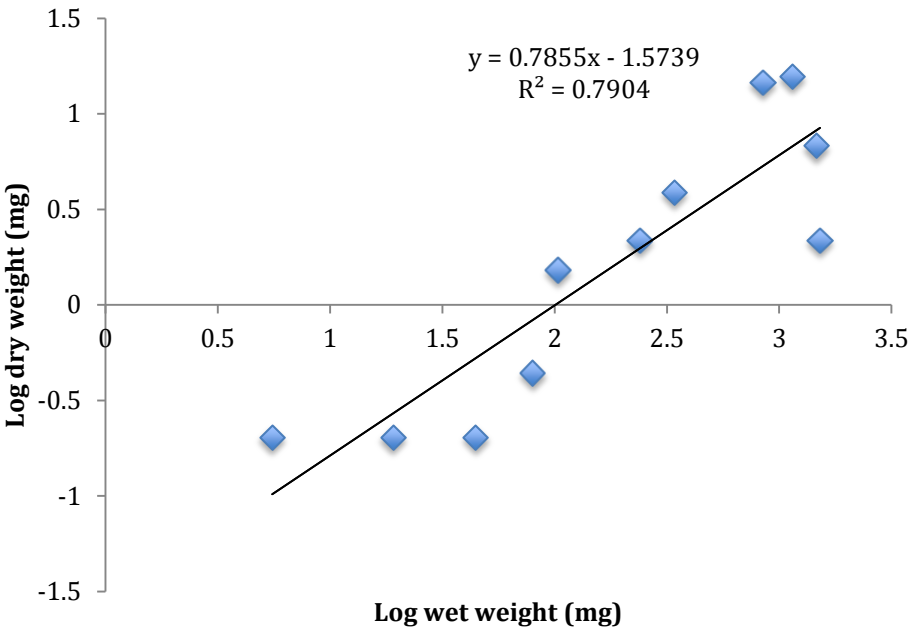

Figure 3: Log-log relationship between wet weight and dry weight for damselflies p<0.001). n=11.

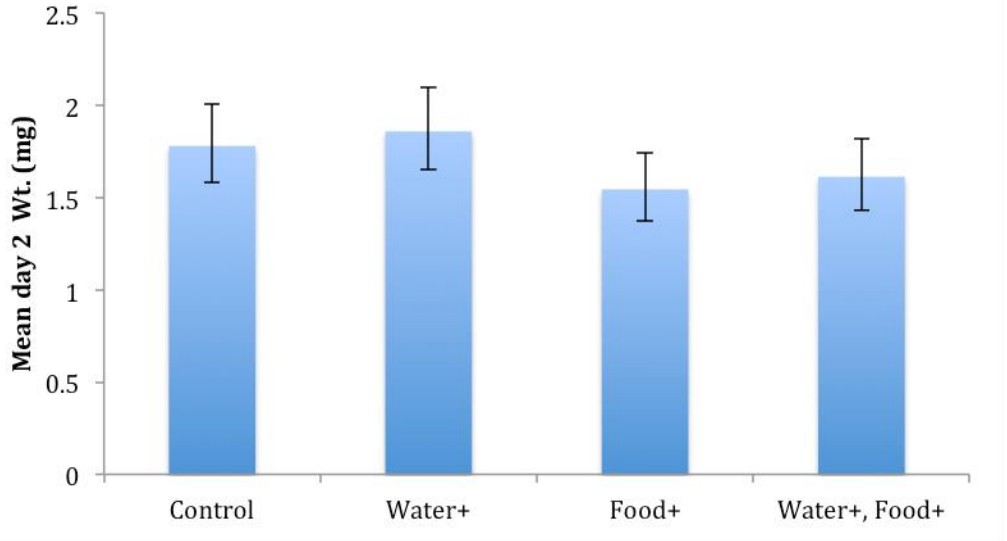

Figure 4: Initial weight is not significantly different between treatment groups (p=0.104). Plotted is the mean dry weight (+/- SE), back-transformed from log-transformation. Control is larvae and prey both grown in 0 µg Cu/L water. Treatment groups are larvae exposed to 2.5mg Cu/L water (Water +), larvae fed with *D. manga* grown in 15 µg Cu/L water (Food +) and larvae exposed to both the water and fed with the copper *D. magna* (Water+, Food+).

Lethal effects

Although mortality did not significantly differ among the treatment groups ($p=0.95$), mortality was only observed in the copper water. 36% of the larvae exposed to copper water died. Mortality started on day 8 and did not differ between food treatment groups. A total of five larvae died, three were in the copper food x water and two in the control food x copper water.

Sublethal Effects

Feeding rates

Copper water significantly decreased feeding ($p<0.0001$) while copper food had no significant effect on food consumption ($p=0.41$, Figure 5). Results are from the last four days. Average consumption for the control was 93% (5.58 out of 6 *D. magna* offered) and was 31% for larvae in the copper water (1.88 out of 6 *D. magna* offered). *D. magna* always died within 24 hours of being placed in the 2.5 Cu mg/L water. However, in the days before feeding was recorded (days 1-6) most larvae were consumed in all treatments.

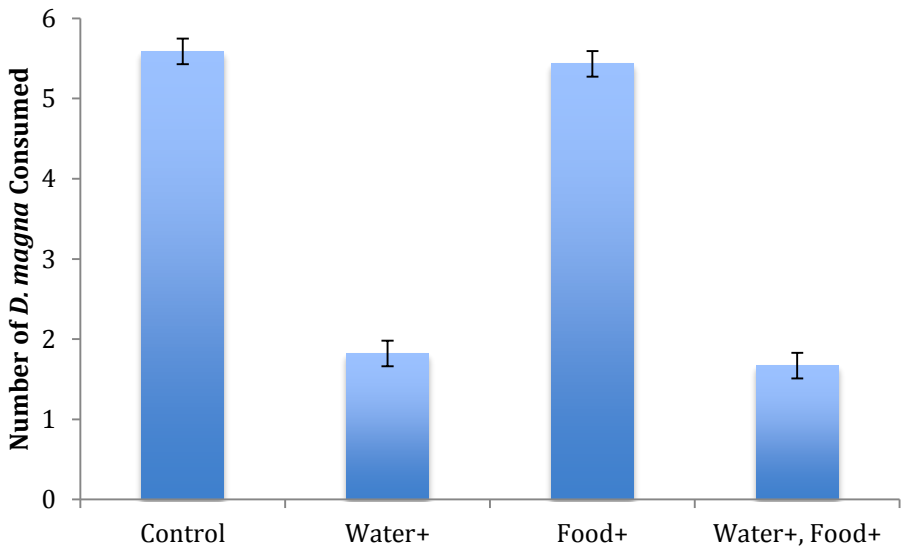

Figure 5: Copper water inhibits feeding in larvae ($p<0.001$). Plotted is the average number of *D. magna* consumed per larvae during the last 4 days (+/- SE).

Molting Effects

The copper diet and copper water had opposite effects on molting: copper water inhibited molting ($p=0.0211$), while the copper food promoted molting ($p=0.0211$, Figure 6). On average, 26.7% of the control larvae molted (95% CI 6.76-64.72%). The copper water decreased this to an average of 1.85% (95% CI 0.11-23.78%). The copper food increased the percent molting to 85.57% (46.26-98.29%). The effects of copper in the food and water cancelled each other out and the copper food and water treatment molted by the same percent as the control.

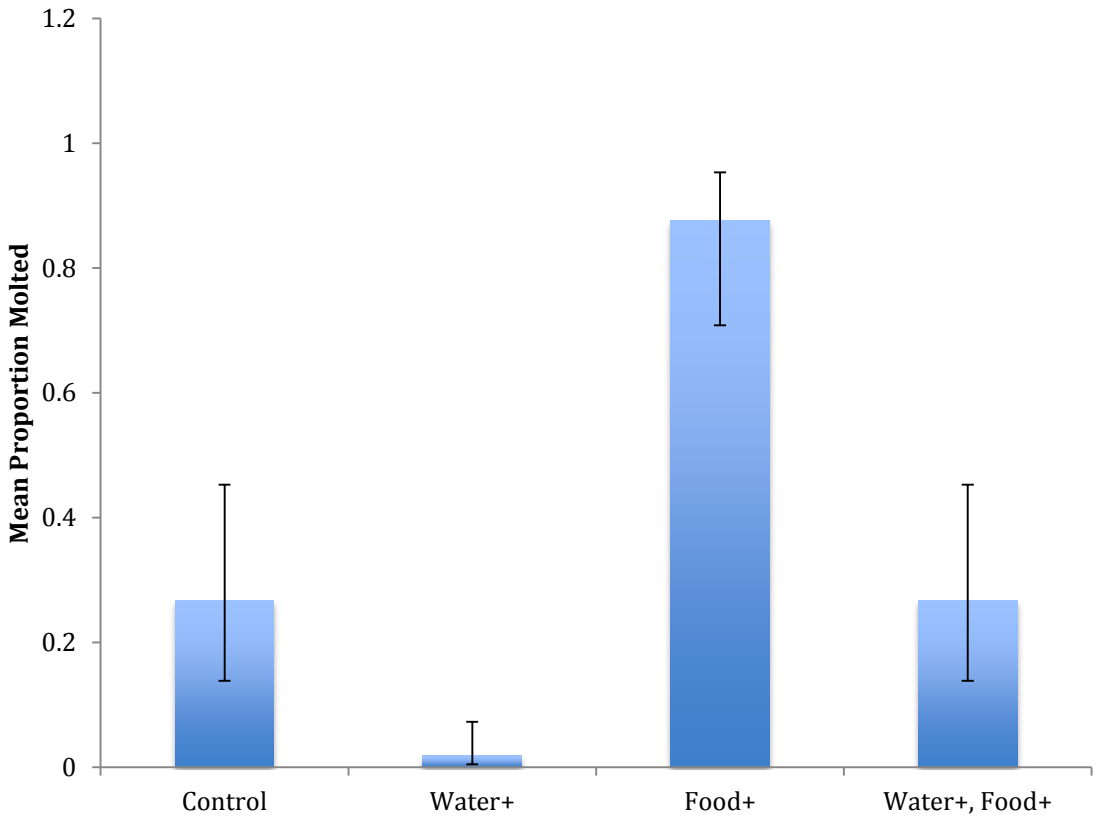

Figure 6: Copper water exposure inhibited molting, while copper food treatment promoted molting (p=0.0211). Plotted is the average probability of molting (+/- 1 SE) given their treatment (n=7). Control is larvae and prey both grown in 0µg Cu/L water. Treatment groups are exposure to 2.5mg Cu/L water (Water +), larvae fed with *D. manga* grown in 15 µg Cu/L water (Food +) and larvae exposed to both the water and given the *D. magna* in copper (Water+, Food+).

Larval Growth

The copper food increased growth (p=0.025, Figure 7) and was the only significant influencer on growth. Larval growth during the 10-day exposure varied both within treatments and across treatments. Average growth rate in the control was 0.002 1/day (95%CI: -.004 to .008 /day). The average larvae exposed to copper water and control food did not grow, and lost weight with average growth at -0.000078 1/day

(95%CI -0.0065 to 0.00634). The additive effect of copper food and water was a growth of 0.0173/day (95% CI 0.0109 to 0.0257µg/day).

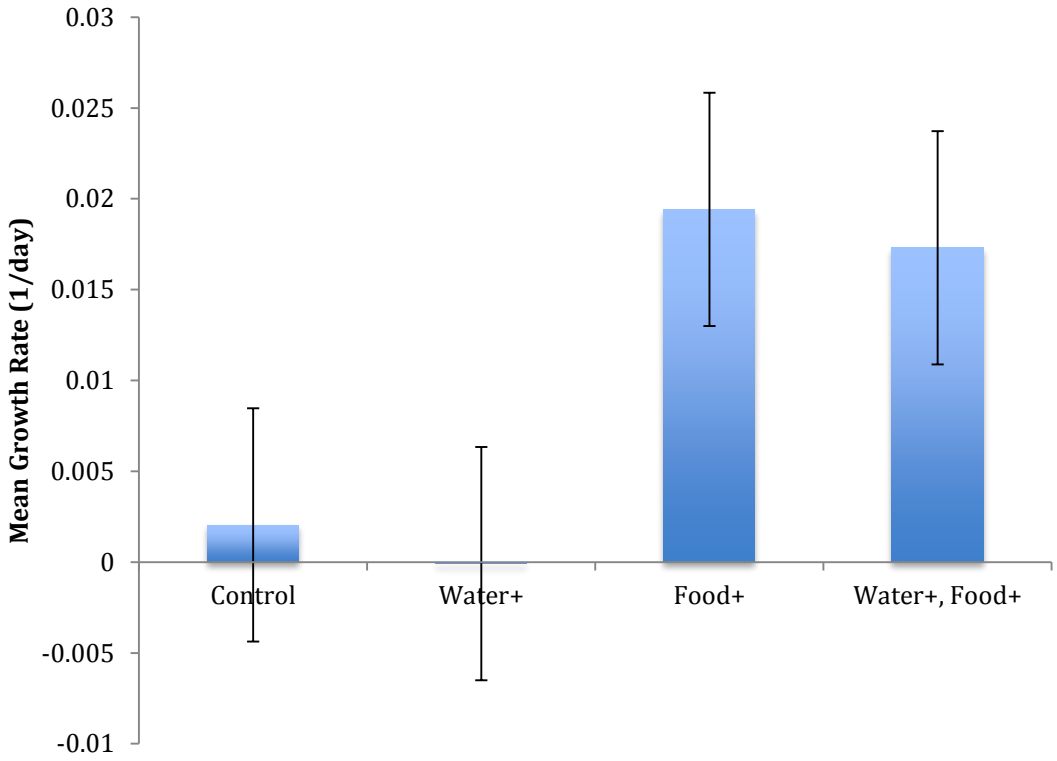

Figure 7: Copper food increases average larval growth (+/- 1 SE). Growth is measured by change in larval dry weight (mg) over a 10-day trial. Larvae exposure to 2.5 mg Cu/L (Water+) has a negative average growth, but growth was only a significant effect for larvae fed the copper treated *D. magna*, grown in 15 µg Cu/L (Food +, p=0.025).

Copper Body Burden

Larvae accumulated copper in their tissue from the copper water ($p<0.0001$) (Figure 8). Body concentration of larvae in the copper water treatment was approximately 2777µg Cu/g dry weight (95% CI: 1892-4063). Only the water exposed groups were over the detection limit of AAS (0.077 ppm). After accounting for body mass, the relationship

between growth rate and copper body burden was negative but only marginally significant (Figure 9, p=0.15).

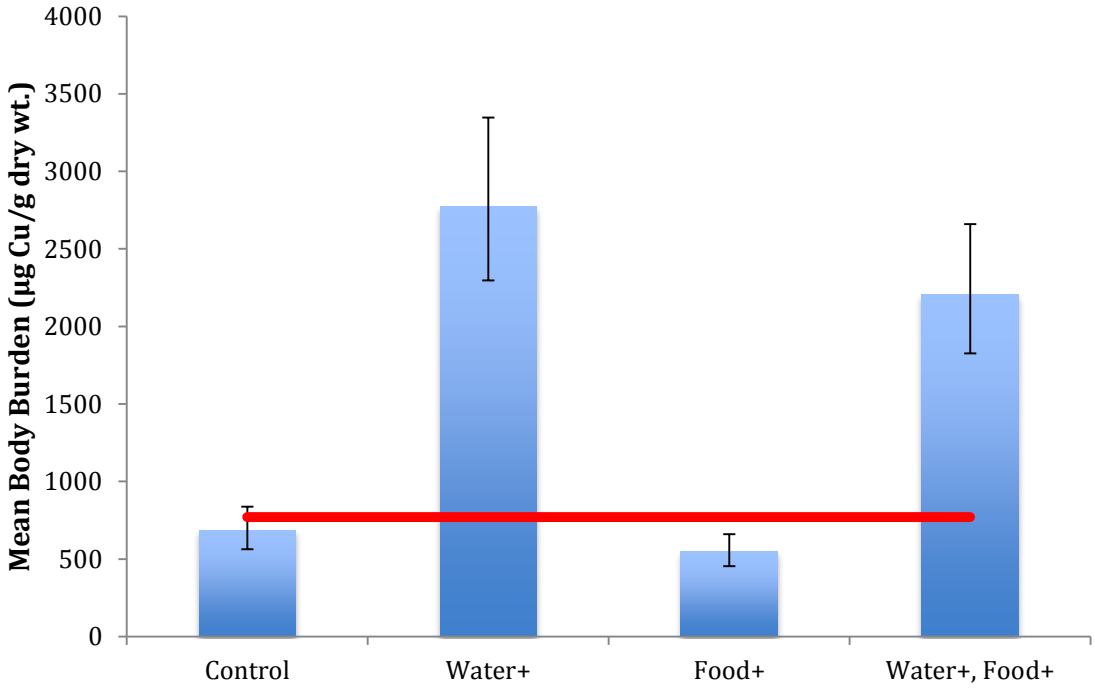

Figure 8. Larvae accumulated copper in body from copper water, whereas larvae had a lower average body burden when given copper food. However, detection limit is 770µg Cu/g dry wt (red line). Log transformed average body burden (+/- 1 SE) is plotted from the copper concentrations of whole-body samples of larvae in each treatment (n=7).

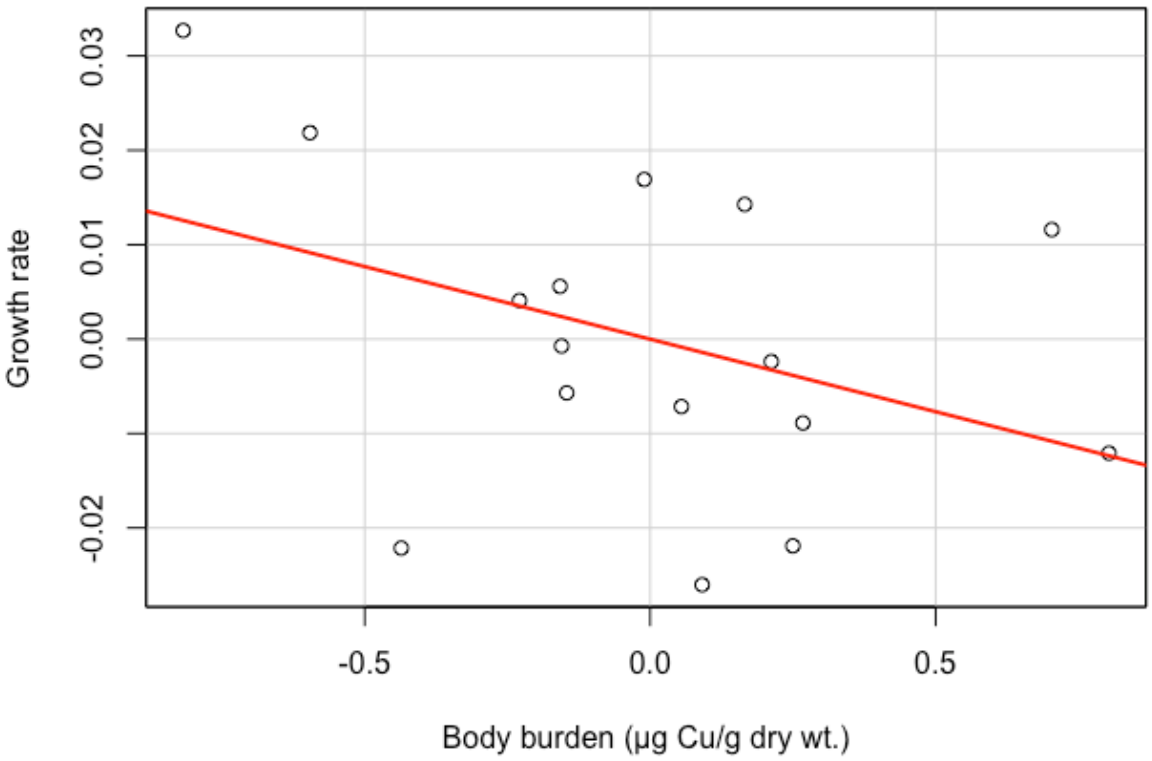

Figure 9: After accounting for initial mass, copper body burden depresses growth (p=0.16). The correlation between copper body burden and growth rate is plotted with the added variable of mass.

DISCUSSION:

Aqueous exposure to Cu inhibited molting and increased body burden of copper in *Ischnura sp.* (Odonata: Coenagrionidae). Although these changes did not result in significantly different growth rates, those larvae exposed to aqueous Cu tended not to grow and in some cases lost weight during the experiment. These changes are likely a result of feeding inhibition either due to behavioral changes in *Ischnura sp.* larvae or immobilization of *D. magna* prey in the aqueous Cu treatment. Contrary to our predictions, exposure to prey grown in Cu-treated water did not significantly change the body burden of Cu in the damselfly larvae. Instead we observed significantly higher growth rates and molting likely as a result of higher prey consumption in the Cu-treated prey exposure. Together the effects of Cu exposure in the prey and ambient water were additive rather than interactive.

Damselflies accumulated high levels of copper to an average body burden of 2770 μg/g dry weight, after being exposed to 2.5 mg Cu/L in water. Average concentration was 2770 μg/L for copper water and the larvae in the control were under the detection limit. This rate of accumulation is similar to the dragonfly larva, *Pachydiplax longipennis*, which accumulated approximately 3000 μg Cu/g dry weight after a 7-day exposure to 2.86 mg Cu/L. The control average body burden was 33.95 μg Cu/g (Tollett et al. 2009). Thus, odonates readily accumulate copper.

Exposure to aqueous copper suppressed development by inhibiting molting. Aqueous exposure did not significantly depress growth. However, the damselflies in the copper

water did tend to exhibit negative growth rates on average. Furthermore, copper body burden depressed growth, but trend was marginally significant. Copper accumulation depresses growth in a variety of freshwater organisms, including many species of fish (Woltering 1983) and invertebrates (Beasley & Kneele 2002). Molting is a less studied endpoint, but copper decreased molting frequency in tiger shrimp, *Penaeus monodon,* at a concentration of 0.90 mg/L (Chen & Lin 2001).

Pesticides have also been shown to inhibit development (growth and molting) in odonates. Odonate growth decreased with pesticides: dichlorvos (Van Praet et al. 2014) and chlorpyrifos (Janseens and Stoks, 2013). The pesticide, chlorpyrifos, accelerated metamorphosis in the damselfly, *Enallagma cyathigerum*, when given ample amounts of food. However, when food was limited the exposure to the pollutant decelerated development. The authors attributed the deceleration as an indicator of the energy cost of detoxification (Janseens & Stoks 2013). Accelerated growth also depresses energy reserves in odonates (Stoks et al. 2006a). Thus, there might not be enough energy available for growth when an organism is deprived of food and exposed to a pollutant. In our study, the combination of both reducing prey availability and diverting energy from growth to detoxification likely interacted to cause depressed growth and development in *Ischnura sp*.

Exposure to the copper via the prey we used in the experiment, *D. magna*, had opposite effects on the larvae. Dietary exposure to Cu seemed to promote growth and molting two obviously related endpoints. All damselflies that molted had positive growth in the experiment. Prior to molting, damselflies decrease feeding and feeding increases

immediately after molting (Corbet 1980). In our study, the mass dropped one to two days prior to molting and increased after molting. Thus, in our experiment growth was likely higher as a result of the different feeding rate during the intermolt rather than a negative effect from accumulating copper from the *D. magna*. The copper food did not affect body burden. All of the damselflies were under the detection limit in the control water and for all the larvae exposed to the copper water the body burden was not greater for larvae given copper treated food than larvae given control food. The copper treated *D. magna* was also under the detection limit. The *D. magna* were grown in a low concentration to ensure survival. This level could be insufficient to be accumulated in odonates.

The two different concentrations to which the prey and the damselfly larvae were exposed (*D. magn*a grown in 15 μg/L, *Ischnura sp*. exposed to 2.5 mg/L) could have led to the different responses we observed in the experiment since the energy costs vary for the two exposure routes. Aqueous exposure killed the *D. magna*, reducing the prey availability. Thus, feeding rates were lower and less energy was available to molt, eliminating the chance to remove the pollutant by molting. Metamorphosis was accelerated by chlorpyrifos when given ample food, but decreased under low food (Janseens & Stoks 2013). This suggests that responses to pollutants vary depending on the amount of energy available. Odonates accumulate metals in the exoskeleton (Hare 1992). Thus, molting could be a defense to remove the copper, but due to the energy cost in the aqueous Cu exposure, larvae did not have enough energy available to molt. One of the two damselflies that molted in the aqueous Cu treatment died within the same day of

molting. This suggests that the larvae in the copper water did not have enough energy to molt as result of decreased prey availability.

Feeding inhibition and growth reduction, led to lethal effects in the 2.5 mg/L exposure. Mortality for the dragonfly, *P. longipennis*, occurred at lower concentrations than those we observed in this experiment 150μg/L (Tollett et al. 2009). This concentration is unlikely to be in any ponds in the field, but can exist in mining ponds (EPA 2015). Thus, damselflies are tolerant to copper. Lethal effects only occurred after day 8 at the high concentrations we used in this experiment. Similarly, feeding also decreased from day 7 to day 8 with few larvae eating at all after day 8.

As the lethal effects were not immediate, they are not likely directly caused by the pollutant. Rather, death resulted from starvation caused by unavailable food (mechanism 1) or due to the energy cost of detoxification (mechanism 2). In both cases this would result in less available energy for growth and molting as well as activity such as feeding. The ultimate result in both cases is metabolic arrest and death.

In order to determine the mechanism that contributed to the depressed feeding and molting in odonates, I recommend a future study that uses a prey species more tolerant of copper exposure, thereby reducing the confounding variable of food availability in this study. The midge, *Chironomus tentans*, can survive at a much higher copper concentration than *D. magna* with an LC50 of 977μg/L (Gauss et al. 1985).

This study reveals that copper decreases development and reduces feeding from prey of daphnids at 2.5 mg/L. The feeding inhibition is either caused by aqueous Cu killing prey and reducing prey availability *or* aqueous Cu increasing the copper body burden and

decreasing feeding rate independent of prey availability. In the event that feeding inhibition was simply because less food is available, my study highlights that even highly tolerant species can be indirectly affected by pollution if the pollutant kills their more vulnerable prey. Feeding is reduced with fewer prey, and the odonates will depress growth and molting. This delayed development can have life-long fitness effects even after metamorphosis (Metcalfe & Monaghan 2001). If feeding inhibition is caused by the higher copper body burden rather than the available food, then sublethal effects of reduced feeding can cause growth reduction and decelerated molting, effects that can ultimately cascade to death.

Both mechanisms of feeding inhibition have impacts on the freshwater community. Contaminants either kill their prey or induce a behavioral response of feeding inhibition. By killing their prey, competition will increase and species with a generalized diet will be favored. If the metal induced the behavioral response of feeding inhibition then more resistant species will be favored under frequent contamination exposure.

REFLECTION

The Regis mission "how ought we to live?" embraces the scientific inquiry by being posed as a question. Ever since honors freshman seminar I have heard "start with the evidence" to draw the conclusion from it, rather than start with a conclusion and find evidence to support it. The mission is to discover rather than to prove. This allows us to grow and change our answer with each new experience and piece of information. By studying the sublethal effects of copper on odonates I was able to provide information on effects contaminants have on freshwater ecosystems. Sublethal effects of feeding inhibition and delayed molting helps address how ought we to live with regard to the management of freshwater ecosystems. I also learned the importance of constant questioning and re-evaluation throughout the entire scientific process. From this, I improved part of my study and also learned how to improve it in the future.

The purpose of the pilot study was to ensure that I was using sublethal concentrations. However, during the pilot I also was able to test my methods and improve them for the main experiment. I adjusted my weighing procedures by switching to a light weighing boat, which allowed the scale to measure the larval mass accurately. I also changed the feed for *Daphnia magna* after first culture died. Thus, a good experiment often requires several smaller scale experiments to assess and improve the methods.

Another important step in scientific method is to record other observations than the ones I'm testing. Without recording the feeding rate every day, I was unable to determine whether feeding rate was decreasing as the exposure continued. I remember the larvae

eating more in the beginning of the study, but without recording the values I am unable to run any statistical analysis to determine if it did occur. Since feeding rate effects growth, it could be a confounding variable. Therefore, preparation also involves identifying any potential confounding variables to be recorded during the experiment. Then, a more accurate conclusion can be drawn.

I learned the importance of re-evaluation when I was finishing up the final discussion. I noticed that the control larvae had unusually high copper body burdens. As I was running out of time, I was tempted to just finish my discussion and mention that it was odd without understanding what the result means. However, re-evaluating my methods and discussing the methods I used, how the machine works, and the best procedure in analyzing the samples with a chemist, I was able to fully understand my data. I realized that the calibration curve was inaccurate because all my standard solutions were above the sample concentrations. When I repeated the AA with improved standard concentrations, my calibration curve resulted in plausible results. This changed my conclusions as all the control water larvae all under the detection limit. Thus, if I hadn't gone back to figure out the cause of the erroneous result, my conclusion would be inaccurate.

In essence, you can't go through the motions of science. It demands time to understand each step in the procedure and all potential variables. Prior knowledge is important but also going back and evaluating each step after the data is beneficial because I was able to fix one of the problems in the data and now have a better idea on a procedure for a future study. In order to ensure that the conclusion is accurate instead of

the result of a procedure error, understanding the methods prior to the experiment and re-evaluation after the experiment is required. In other words, slowing down to completely understand what I was doing and why ultimately determined the quality of the end result.

My thesis contributes to understanding sublethal metal pollution. Damselflies are very tolerant to metal pollution but were affected by sublethal effects of decreased molting and feeding. Therefore, even tolerant species are vulnerable to metal pollution because the contaminant can kill their prey and decrease prey availability or cause a behavior effect of feeding inhibition, which can lead to starvation. The EPA monitors for freshwater toxicity by creating a criteria to prevent lethal losses. However, sublethal concentrations can eventually lead to death. Therefore, sublethal effects should be included in the criteria in order to assess the freshwater ecosystem.

Freshwater ecosystems provide a variety of services to humans. As new knowledge of ecosystem services and our integration in freshwater ecosystems is provided, so should our treatment of them. Aldo Leopold argued that we need to evolve our ethics to include biotic community and to judge an action "right when it tends to preserve the integrity, stability and beauty of the biotic community. It is wrong when it tends otherwise" (Leopold 1949). In order to promote the integrity and stability of ecosystems, re-evaluation of management policy is needed as new information of pollution effects is provided. Thus, science and the constant inquiry of "how ought we to live?" can help us understand what we are doing and why which allows us to improve and grow, becoming better members of our biotic community.

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