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Effects of Predatory Cues on Copper Sensitivity and Metabolic Rate in Gambusia affinis

Melissa N. Campbell
East Tennessee State University

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Effects of Predatory Cues on Copper Sensitivity and Metabolic Rate in *Gambusia affinis*

A thesis presented to

the faculty of the Department of Biological Sciences

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by

Melissa Campbell

May 2017

Dr. Joseph R. Bidwell, Chair

Dr. Fred Alsop

Dr. Karl Joplin

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ABSTRACT

Effects of Predatory Cues on Copper Sensitivity and Metabolic Rate in *Gambusia affinis*

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Aquatic organisms are subject to a number of stressors from natural (temperature, etc) and anthropogenic origins (pesticides, etc). Natural stressors may amplify the effects of contaminants and increase an organism's sensitivity to them. This study sought to examine how predatory cues (kairomones & alarm cues) affect copper tolerance in the mosquitofish, *Gambusia affinis*. Fish metabolic rate was evaluated to gain insight on any interactive effects between predatory cues and copper. Paired 96-h toxicity tests with copper and predatory cue and with copper alone were conducted to determine the influence of predatory cues on *G. affinis* metal sensitivity. In the presence of alarm cue neither *G. affinis* copper tolerance or metabolic rate was altered. At copper concentrations ranging from 0.25mg Cu/L - 0.50mg Cu/L kairomone presence had no influence on survival, while between 0.50mg Cu/L and 1.0mg Cu/L, kairomone presence increased survival. Kairomone had no significant effect on metabolic rate.

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CHAPTER 1

LITERATURE REVIEW

In aquatic environments, organisms are subject to a variety of stressors which originate from both natural and anthropogenic sources. Fluctuations in daily temperature, heavy rain events, food availability, and the presence of other organisms are all examples of natural stressors which may affect an organism's physiology and behavior. An animal's response to these stressors depends on the organism in question, the stimuli present, and even the environmental conditions (Holmstrup et al. 2010). The introduction of human activity further adds to the complexity of aquatic environments. Physical changes such as water withdrawal, damming, and recreational use, as well as chemical inputs such as pesticide and metal contamination, are examples of anthropogenic stressors which may affect aquatic organisms (Ometo et al. 2000). The presence of these anthropogenic stressors, especially contaminants, often has deleterious effects.

Traditional toxicological studies focus on a single contaminant and the physiological responses it produces in an organism (Robison, 2010; Melvin and Wilson, 2013). It is well established that severity of these effects vary, and abiotic factors such as temperature, pH, water hardness, and time of exposure affect an organism's sensitivity to contaminants (Pistole et al. 2008; Pourkhabbaz et al. 2011; Schlomer, 1999). For example, hypoxic conditions combined with contaminant presence have been shown to increase copper sensitivity in shore crabs *(Carcinus maenus)* (Holmstrup et al. 2010) and zinc sensitivity in the common carp, *(Cyprinus carpio)* (Hattink et al. 2006). Sih et al. 2004 stated that future laboratory studies should incorporate the effects of multiple stressors to better simulate real-world conditions. In the last few decades interest in the potential interactions between biotic stressors and contaminants has been growing. Research in amphibians, insects, and some fish, have looked into the possible synergistic effects of pesticides and heavy metals in relation to parasites and pathogens (Holmstrup et al. 2010). In addition, research conducted with amphibians has shown that biotic factors, such as predator-induced stress, have the potential to interact with contaminants resulting in physiological and behavioral changes. For example, when exposed to the pesticide carbaryl in tandem with predator cues, gray treefrog *(Hyla versicolor)* tadpoles were more sensitive to the pesticide than those exposed to the pesticide alone. In some trials, the lethality of the pesticide doubled or quadrupled (Relyea and Mills, 2001).

To further investigate this phenomenon, Relyea (2003) exposed six different species of amphibians to predator cues and carbaryl and found that green frogs (*Lithobates clamitans*) and bullfrogs (*Lithobates catesbeiana*) have a decreased tolerance for carbaryl when exposed simultaneously to predator cues. This increase in sensitivity cannot be generalized though, as four other amphibian species showed no synergistic effects of contaminants and predator stress. Interestingly, predator cues increased gray treefrog tadpoles' sensitivity to carbaryl in previous work (Relyea and Mills, 2001), but had no effect on tadpoles in the multiple species study. This suggests not only species-sensitivity, but population differences (Relyea, 2003).

The synergistic effects noted in organisms may stem from general stress and stressors. Predation cues may initiate an acute alarm response as organisms attempt to combat the stressor or escape it. An increased metabolic rate is indicative of the initial "fight or flight" response (Selye, 1956) and the resulting phases which occur are part of the general adaptation syndrome (GAS) (Selye, 1956). Stimuli which trigger the stages of the general adaptation syndrome, or "stressors," may be biotic or abiotic, natural or anthropogenic in origin. While the GAS is beneficial in the short term, aiding in escape from possible nearby predators, prolonged expression results in negative results. The severity of the stressor, whether it is a chronic or acute, and whether it originates from multiple or single sources all contribute to how an organism may cope (Campero et al. 2007; Janssens and Stoks, 2013). Clinchy et al. (2013) found that sustained presence of stressors and the resulting effects on the physiology and behavior of an animal become detrimental.

When stress is chronic, for example in areas with high-predator density, energy stores may be reallocated in an organism. Sustained increases in metabolic rate and shifts in energy budget in the presence of predator and alarm cues can result in low growth rates, morphological changes, and other behavioral shifts (Selye, 1976; Ferrari et al. 2010). Similar responses have been noted from stressors such as contaminants. From this, it is reasonable to suggest that responses of aquatic species may be altered by the presence of both contaminants and predator-stress. While research has investigated how contaminants affect responses to predators following contaminant exposure (Mirza and Pyle, 2009; McPherson et al. 2004) few studies have focused on how predatory events affect a fish's sensitivity to contaminants (Robison, 2010).

Natural Stressors

All aquatic organisms must balance the energy used to forage, reproduce, detect predators, and escape from threats. As such, an organism with the ability to detect different warning signals may be more likely to survive and escape predation events. In aquatic environments where chemical cues are easily dispersed, organisms benefit from an efficient ability to detect and respond to the threat of predators. Fish make use of both visual and chemical mechanisms to detect predators. In clear environments, motion and visual cues facilitate predator avoidance (Wisenden and Harter, 2001). In environments where water is more turbid, chemical cues stemming from predators (kairomones) or alarm substances from prey fish themselves (alarm substances/cues) alert other animals to potential dangers (Ferrari et al. 2010). Both cue types are detected via olfaction, but are released in different ways. Kairomones are passively released as water passes over predatory organisms. Alarm substances, however, are only released by prey organisms in a predation event (Chivers and Smith, 1998).

Damage Released Alarm Signals

Chivers and Smith (1998) noted over fifty different species which responded to conspecific cues ranging from aquatic invertebrates to amphibians. In fish, the presence of an alarm substance was first noted in fish of the order Ostariophysi. Known as Schreckstoff, these chemical cues are detected via olfaction and can cause predator avoidance responses (Ferrari et al. 2010; Reed, 1969). These cues may affect a large area (Wisenden, 2008) and it is generally believed that Ostraiophysans' Schreckstoff is a nitrogen-oxide compound such as hypoxanthine-3 (n) oxide (Brown et al. 2000). This alarm substance is completely contained in specialized skin cells, called club cells, and can only be released into the aquatic environment when an individual takes damage (Smith, 1992). When fish detect the alarm cues fish detect the alarm substance, they respond with predator avoidance behaviors (Clinchy et al. 2013).

Although it is difficult to precisely identify compounds that are used for chemical communication, similar, and possibly analogous alarm signals exist in non-ostariophysan fish including some salmonids (Kim et al. 2009) and some cypriondontiformes (Smith and Belk, 2001; Garcia et al. 1992). These compounds are thought to be similar because they illicit a response in the same manner as Schreckstoff as indicated by both laboratory and field experiments (Wisenden et al. 2004; Pfeiffer, 1977). Most studied responses to these skin extracts - here on out referred to as "alarm cue" to differentiate from Schreckstoff cues - consist of behavioral assays which quantify responses of fish in the cues presence (Wisenden et al. 2004; Vanzwoll, 2010).

Predator and Diet Released Cues

Unlike alarm cues, which are released when a prey fish takes damage, kairomones and dietary cues are chemicals passively released from piscivorous predators, but not advantageous for the animal which produces them (Ferrari et al. 2010). Kairomones are thought to be the odor of a fish or the by-product of bacteria associated with the predatory species (Ferrari et al. 2010; Weber, 2003). Dietary cues are chemicals released from the digestion of prey in the waste of predators. Prey that can accurately detect and assess these indicators of predator presence may have an advantage over other organisms.

Studies have investigated the response to predator-derived cues without differentiating between these chemosensory signals (Ferrari et al. 2006). This may be due to the fact that predator-derived cues, regardless of 'type', have been documented to cause both acute and chronic changes ranging from behavioral alterations to morphological changes (Chivers and Smith, 1998; Dodson, 1989; Langerhans et al. 2004). For the purposes of this study, a 'kairomone' will refer to both types of predator-derived cues. Prey species that are able to recognize kairomones are able to assess detailed information. When presented with predation cues derived from northern pike (*Esox lucius*), fathead minnows (*Pimephales* *promelas*) responded more readily to higher concentrations of kairomone. In addition, they were also able to discern predator size (Kusch et al. 2004) and were more readily able to gauge predation risk.

Anthropogenic Stressors

Metal Contaminants

While many studies have demonstrated pesticides can potentially interact with predator cues in aquatic organisms, few, if any, have investigated how contaminants such as heavy metals and alarm cues may interact in fish (Scott and Sloman, 2004). This is surprising, as the presence of excess metals in aquatic systems often has negative impacts on fish (Atchison et al. 1987). The majority of studies which investigate predation and metals focus on predator avoidance behaviors (Scott and Sloman, 2004), but other effects are known to exist. In invertebrates, gravid *Daphnia pulex* exposed to copper and predatory cues altered morphology in neonates. During predation events, juveniles from exposure treatments also had much lower survival rates compared to unexposed controls (Mirza and Pyle, 2009). In fish, Iowa darters (*Etheostoma exile)* from metal-contaminated lakes had a decreased response to alarm cues than those from more pristine lakes (McPherson et al. 2004).

Species-specific differences in sensitivity to metals have been described. For example, white sturgeons (*Acipenser transmontanus*) are far more sensitive to copper pollution than rainbow trout (*Oncorhynchus mykiss*) or fathead minnows (*Pimephales promelas*) (Vardy et al. 2013). Cadmium can decrease capture efficiency in multiple predator species (Riddell et al. 2005) as well as cause lethargy in others. Similar effects have been noted in zebrafish (*Danio rerio)* exposed to mercury (Atchison, 1987). Western mosquitofish (*Gambusia affinis*) exposed to mercury are less likely to escape predators. In rainbow trout, cadmium eliminates normal behavioral responses to the presence of alarm cues (Scott et al. 2003). At low concentrations, cadmium changes predator-prey interactions between fathead minnows *(Pimephales promelas*) and largemouth bass (*Micropterus salmoides*) (Sullivan et al. 1978).

Copper Contamination

Copper (Cu) is an essential metal needed for an assortment of biological pathways but in excess becomes toxic to organisms. Increased concentrations of copper may be attributed to human disturbance and anthropogenically-derived copper may arrive in waterways via runoff from urban areas or from manufacturing waste (Schlomer, 1999). Copper may be taken up by fish either from dietary sources or passively through gill epithelial cells. Excess copper may accumulate in the kidneys and liver of fish. Copper can alter organisms' behavioral and physiological response to secondary stressors.

Colorado pikeminnow (*Ptychocheilus lucius)* exposed to copper for acute durations expressed inhibited responses to alarm substances, and at some concentrations, complete loss of response (Beyers and Farmer, 2001). In Atlantic silversides *(Menidia menidia),* schooling behavior increased in the presence of copper and in bluegill (*Lepomis macrochirus*), social behavior was also altered (Scott and Sloman, 2004). A review by Atchison et al. (1987) noted other behavioral changes in fish including hyperactivity and feeding depression. In bluntnose minnow *(Pimephales notatus),* the presence of copper altered growth rates in young and reduced reproduction (Horning and Neiheisel, 1979). Juvenile fathead minnows exposed to copper during development were unable to accurately recognize conspecific alarm substances in fish (Carreau and Pyle, 2005). It has also been established that genetic variation and the environment an organism originates from also contribute to metal tolerance (Bendis and Relyea, 2014; Annabi et al. 2009). As such, this project aims to use a novel model species to investigate if possible interactions from natural stressors (kairomone and alarm cues) and an anthropogenic stressor (copper) occur during concurrent exposure.

Model Species

The study organism for this project is the mosquitofish (*Gambusia affinis*) - a small, highly abundant freshwater fish. These fish are livebearers, generally silver in color, and range in size from 1 cm - 6 cm (Pyke, 2005). Wild fish are omnivores eating plant-based materials to aquatic invertebrates. Mosquitofish are historically native to the southern and eastern portions of the United States; however, two sister species, the eastern mosquitofish (*Gambusia holbrooki*) and western mosquitofish (*Gambusia affinis*), have spread around the globe (Pourkhabbaz et al. 2011). This is due widespread introduction to ponds and slow-moving waterways for mosquito control. Both species are easily reared in laboratory conditions, and may be obtained commercially or captured from wild populations.

Location of origin can affect *G. affinis'* sensitivity to metals (Annabi et al. 2009) as well as the genetic diversity of the population (Chagnon and Guttman, 1989). In addition, an organism's life history and previous exposure to predators increases their likelihood to respond to predator cues (Ferrari et al. 2010). *G. affinis* offer a unique advantage for this project due to the abundance of individuals in a natural population near the location of this project and have experience with predatory sunfishes (*Centrarchidae*) inhabiting the same location (Page and Brooks, 2011). Another advantage to using *Gambusia* is their ability to respond to injured individuals of the same species (Garcia et al. 1992). While these alarm cues have not been characterized (Pyke, 2005) *Gambusia* respond to their presence with predator avoidance behaviors. These include reduced movement, changes in vertical distribution, and schooling (Bryant, 1986; Garcia et al. 1992). While there is little evidence that demonstrates precisely how alarm cues affect *Gambusia* physiology, some research has looked into reproductive response (Vanzwoll, 2010).

Predation Events and Copper

Numerous factors may affect responses to alarm cues and kairomones, including group size, time, and the species involved (Ferrari et al. 2010). Fish responses to copper are also subject to biotic and abiotic factors but many alter energy needs (Saez et al. 2013). Predation stress is considered to elicit reactions attributed to the "fight or flight" response (Selye, 1956). Behavioral changes (i.e. increased sensitivity to motion, freezing and erratic swimming), enzymatic activity, and increased metabolic rates (Robison, 2010) are all indicators of a flight or fight response in fish. Therefore, physiological changes resulting from the presence of predation cues may interact with changes due to contaminants. If the presence of cues are indeed characterized by energy reallocations (Slos and Stoks, 2008) this may be indicated in changes in metabolic rate. Oxygen consumption offers a quantifiable measurement of such changes in regards to a whole animal response. If the alarm cue in *G. affinis* initiates the GAS, we expect to find higher metabolic rates in fish exposed to alarm cues than those who have not been presented with the cues. Additionally, no studies with *G. affinis* have attempted to investigate predatory cues and metal contamination.

CHAPTER 2

EFFECTS OF PREDATION CUES ON COPPER SENSITIVITY AND RESPIRATION IN THE WESTERNMOSQUITOFISH (*Gambusia affinis*)

Introduction

Organisms living in aquatic environments are subject to a number of challenges that can impact their ability to mature and reproduce. Natural stressors, such as temperature, drought, and food availability affect how aquatic organisms balance their energy needs and expenditures. This can alter both physiology and behavior (Ometo et al. 2000). In addition to natural stressors, increased human activities such as habitat modification and contamination often result in detrimental effects to aquatic organisms (Campero et al. 2007). The understanding of how these combined factors affect organisms is essential for the practical management of anthropogenic contaminants.

Conventional toxicity testing has been used extensively to test the adverse effects of anthropogenic contaminants. These tests often focus on a single species and single pollutant and may not accurately reflect real world conditions (Melvin and Wilson, 2013, Robison, 2010). In recent years, researchers have acknowledged the limitations of these tests which has resulted in a shift towards experiments that investigate the interactions between multiple environmental factors (Sih et al. 2004). For example, shore crabs (*Carcinous neanus*) and common carp (*Cyprinus carpio*) have been shown to have an increased sensitivity to copper and zinc, respectively, in hypoxic conditions (Hattink et al. 2006; Holmstrup et al. 2010). *Gambusia affinis* exposed to copper, zinc, and nickel are more sensitive to metals as water hardness decreases (Kallanagoudar and Patil, 1997). Temperature, pH, and other abiotic factors also influence organismal responses to contaminants (Pistole et al. 2008; Pourkhabbaz et al. 2011).

Studies which focus on how biotic factors like predatory cues, such as kairomones (passively released cues from predators) and alarm cues (damage released chemicals detectable to conspecifics) have increased in the past decade (Clinchy et al. 2013, Ferrari et al. 2010, Wisenden, 2008). For example, the presence of copper has been shown to impair morphological defenses in *Daphnia pulex* and decreased survivorship in the presence of a predator (Mirza and Pyle 2009). Interactive effects between stressors are well documented in invertebrates (Campero et al. 2007, Qin et al. 2015), however, research using vertebrates is less prevalent.

Research on vertebrates which does exist focuses largely on amphibians and pesticides (Holmstrup et al. 2010, Relyea, 2012, Relyea and Mills, 2001). Potential interactions between predatory cues and contaminants are likely not limited to amphibian models. Work with fathead minnows (*Pimephales promelas*) exposed to cadmium showed that the presence of kairomone did not affect their survival. Conversely, kairomone did increase minnow sensitivity to NaCl (Robison, 2010). This suggests that multiple stressor interactions are variable and warrant further study.

In vertebrates, it has been suggested that the physiological stress resulting from the combined exposure to both predatory cues and contaminants may be mechanism for documented synergistic interactions (Relyea, 2003). This "combined physiological stress" may result from the 'fight or flight' response (Selye, 1956) in which physiology and behavior may be acutely altered to handle stimuli. However, sustained reallocation of energy in this state has been shown to be detrimental to an organism (Clinchy et al. 2013). Both contaminants and predator presence have been shown to initiate changes in morphology, behavior, and respiration (Beyers and Farmer, 1999; Carreau and Pyle, 2005; Scott and Sloman, 2004). As such, if the 'fight or flight' response has been initiated there may be a measurable change in metabolic rate that could be used to indicate a general stress response.

This project's goal was to determine if the presence of conspecific alarm cues and kairomones (derived from predatory bluegill *Lepomis macrochirus)* influenced the sensitivity to copper in the mosquitofish, *Gambusia affinis*. This was done by measuring the mortality of adult *G. affinis* to copper in the presence and absence of predatory cues. Furthermore, metabolic rate (indicated by oxygen consumption) was examined to investigate the potential role which the general stress response plays in for any observed combined effects in toxicity bioassays. The western mosquitofish was used as a model due to its abundance across the globe (Pourkhabbaz et al. 2011). In addition, *G. affinis* are known to respond to injured conspecific skin extracts with predator avoidance behavior (Garcia et al. 1992). Copper chloride ($CuCl_2$) was used as the contaminant of interest as copper is essential for biological processes, but has deleterious effects when present in excess and can impair of olfactory abilities, alter behavior, and disrupt ion regulation (Beyers and Farmer, 2001; Carreau and Pyle, 2005; Scott and Sloman, 2004). Copper may enter aquatic systems in a number of ways. Copper is used in numerous products (e.g. machinery, fertilizers) and can enter waterways from urban runoff, manufacturing waste, and smelting (Eisler, 1998; Schlomer, 1999)

Toxicity Bioassay Hypotheses

1) In 96-h toxicity tests, adult *G. affinis* survival will significantly decrease when presented conspecific alarm cues in tandem with copper chloride compared fish exposed to only copper chloride.

2) In 96-h toxicity tests, adult *G. affinis* survival will significantly decrease when exposed to copper chloride and predator conditioned diluent water (bluegill kairomones) simultaneously compared to copper chloride exposure alone**.**

Metabolic Rate Hypotheses

1) Adult *G. affinis* oxygen consumption will be higher in individuals exposed to conspecific alarm cues than those exposed to moderately hard water alone.

2) Adult *G. affinis* presented with bluegill kairomone will have increased oxygen consumption compared to fish presented with moderately hard water alone.

Behavioral Hypothesis

1) The presence of conspecific alarm cue will increase the index of cohesion (group closeness) exhibited by *G. affinis* compared to fish exposed to controls.

Methods

Test Organisms

Adult *Gambusia* were collected by seine and dip-netting from Phipps Bend Wildlife Management Area in Hawkins County, Tennessee, from April 2015-July 2016. Fish were transported to an approved animal holding room in Brown Hall B27 at East Tennessee State University (ETSU). The holding room temperature was kept between 21-22°C, which was also the temperature at which experiments were conducted. When field water temperatures were lower than the holding temperatures, fish were maintained in 75-L glass aquaria in an environmental chamber set to field conditions. To transition fish from cooler field conditions to the holding temperature, the temperature in the 75-L aquaria was raised 1°C a day using submerged heaters until the holding temperature was reached. Following acclimation, fish were moved to clean 75-L glass aquaria for one week to identify any individuals that appeared to have compromised health (abnormal behavior, deformation, parasites, or bacterial infections). Any animal with indications of compromised health was removed from the tanks and humanely euthanized by immersion in 250 mg/L Tricaine Methanesulfonate (MS-222) (AVMA 2007). When water temperature in the field did not vary from laboratory conditions, new fish were directly placed into clean 75-L aquaria and acclimated for one week. Following the quarantine period, individuals were separated into another set of 75-L glass aquaria maintained at 21°C- 22°C with submersible heaters for an additional two weeks before use in experiments. During holding *G. affinis* were fed twice daily with Tetramin Fish Flakes (Spectrum Brands, Blacksburg, VA, USA). Prior to use in bioassays, fish were fasted for 48 h and not fed during experiments.

Additionally, bluegill sunfish (*Lepomis macrochirus*) used to produce predator cues were collected using dip nets from a private pond located in Unicoi County, Tennessee. Individuals (>12cm, 40-70g) were transferred to ETSU and housed in a 378-L glass aquarium at 21°C containing aerated, dechlorinated tap water and equipped with a submersible sponge filter and heater. Fish were acclimated for two weeks prior to use for predation cue collection. Bluegill were fed Aquamax Sport Fish 600 Fish

Food Pellets (Purina Mills, Gray Summit, MO, USA) once a day when not used for predator-derived cue collection. During capture, housing, and experiments, all animals were kept and utilized according to protocols approved by the East Tennessee State University Animal Care and Use Committee (Protocol P150201).

Preparation of Predation Cues

Kairomone (K in results) was obtained from four adult bluegill chosen at random from the laboratory colony. Each fish was weighed, and individually separated into four 9-L glass aquaria containing moderately hard water (MHW, USEPA, 2002). Bluegill were loaded at a rate of 10g/L MHW (Spivey et al. 2015). Each fish was fed a single *G. affinis* and left to soak for 24 h after which they were removed and the predator-conditioned (kairomone) water was transferred into a single 19-L polyethylene canister (Uline, Pleasant Prairie, WI, USA). Kairomone water was stirred using an aquarium net used exclusively for this purpose for five minutes to create a homogenized mixture and then filtered (filter: 5μm pore size, 92 cm x 183 cm, Duda Energy, Decaunter, AL, USA) to remove debris and other organic material. Filtered kairomone water was used in the kairomone bioassays within 60 min. This procedure was repeated every 24 h over the course of the 96-h toxicity tests to maintain fresh cue during tests. Identical methods were used to collect treatments for respirometry experiments.

Alarm cue (AC in results) was collected from adult *G. affinis* selected haphazardly from the stock population. Fish were sacrificed using cervical dislocation followed by brain pithing (AVMA, 2007). Two collection methods were used depending on the experiment the alarm cue was used in. To procure alarm cue for behavior trials, 10 shallow lacerations were made on each side of a single adult *G. affinis* euthanized as described above. The lacerations were deep enough to break the epidermis but not cause bleeding. Each side of the fish was then rinsed with 10 ml deionized water into a clean 50-mL glass beaker (Nordell 1998). The solution was then filtered through filter paper (2.5 μm pore size, 10 cm diameter, Fisher Scientific, Fairlawn, NJ, USA) to remove scale and debris and used within 60 min of collection.

To produce the larger volumes necessary to conduct toxicology and respirometry work, alarm cue was collected using a different procedure. Skin from each side of the specimen was removed with care taken to avoid inclusion of any muscle or connective tissue. A total of 10 cm^2 skin (n=4-6) was collected for each stock solution of alarm cue. Skin fillets were kept moist during collection and then added to 50 mL of distilled water. The skin extract and solution were transferred into 1.5mL Eppendorf tubes (Fisher Scientific, Fairlawn, NJ, USA) and homogenized using a Bullet Blender Throughput Bead Mill Homogenizer (Next Advance, Averill Park, NY, USA) at a speed of 12 for 1 min and 10 for 2 min. Eppendorf tubes were then moved to a microcentrifuge (BIO-RAD, Hercules, CA, USA) and run for 20 minutes at 4000rpm (1308 x g). The supernatant was decanted and poured into a 100-mL beaker through Fisher filter paper (2.5μm pore size, 7cm diameter, Fisher Scientific, Fairlawn, NJ, USA) to remove remaining particles. The solution was then diluted to a final concentration of 0.1cm^2 skin homogenate/mL deionized water (Pollock et al. 2003). The stock solution was separated into 30-ml aliquots and stored in 50 ml polyethylene tubes (Fisher Scientific, Fairlawn, NJ, USA) at -4°C until use within 30 days.

Behavioral Response to Alarm Cues

Behavioral assays were conducted to determine if *G. affinis* responded to alarm cue by increasing group cohesion (Vanzwoll, 2010). Twelve fish were randomly assigned to one of two 40-L glass aquaria (one control treatment and one cue treatment) with 6 individuals per tank and allowed to acclimate for 24h. To measure group cohesion, a white background containing a grid of 5cm x 5cm squares was attached along the length of each aquaria. The treatment (AC or MHW) was introduced through a piece of aquarium airline tube glued to the wall of the aquarium and extending approximately 45cm into the tank. An air stone attached to an aquarium pump by airline was also placed in each tank approximately 50mm below the cue line to facilitate mixing following cue introduction. Dispersal of cue in the tank was first characterized by injecting red food coloring through the delivery tube above the air stone. It was determined that nearly complete dispersal occurred throughout the tank in approximately 2 min. A total of ten trials were conducted (5 per treatment). Test aquaria were blocked from view of each other and from external stimuli using a corrugated cardboard barrier. Within this barrier, two Go-Pro cameras (GoPro Inc, San Mateo, CA, USA) were positioned on a 2-cm high stand situated 12cm from an aquarium. Each behavioral trial lasted 8-min and included a 2-min pre-stimulus phase, introduction of the cue, and a 6-min post stimulus phase. For cue introduction, the control aquarium received 20mL of deionized water through the injection port while alarm cue treatment received 20mL of cue. For the duration of the trial, a photo was taken every 30 seconds to record the position of the fish in each aquarium with reference to the grid described above.

Metabolic Rate Response to Predation Cues

Routine metabolic rate (RMR) is the post-absorptive, resting metabolic rate in fish which takes into account random activity and the maintenance of functions (Lindgren and Laurila, 2009). RMR was determined for individual fish in the presence of kairomone, alarm cue, or a MHW control. All trials were conducted at 21°C in an incubator (ThermoScientific, Waltham, MA, USA) using intermittent flow-through respirometry.

A water bath was placed within the incubator to further control temperature on a Cimarec Multipoint Magnetic Stirrer (ThermoScientific, Waltham, MA,USA) used to mix the solutions within the test chambers. 90-mL custom respirometer chambers (Fig. 1) were made from Harvel transparent 40 clear polyvinyl chloride (L = 4.4cm, D = 5cm, USPlastic, Lima, OH, USA) with end caps fabricated from THK copolyester (Eastman Chemical Company, Kingsport, TN, USA). End caps were equipped with C-Flex Clear Tubing for an inlet and outlet tube (1/8"ID x 1/4"OD, Cole-Palmer, Vernon Hills, IL, USA) on opposite sides of the chamber. A single nylon cable fitting (Remora, Barnsley, UK) was placed beneath the outflow to insert the oxygen probe into the chamber. In addition, a perforated copolyester 'floor' (4.0cm x 3.2cm) was placed within each chamber to prevent test organisms from interacting with a magnetic stir rod that was used to facilitate mixing in the respirometry chamber (220rpm).

Figure 1. A partially submerged custom 90mL respirometer

Four chambers were submerged in the water bath for each trial, one for each treatment and an empty respirometer used to measure background oxygen loss. One-way water exchange through each chamber was achieved with a programmable peristaltic pump (Cole-Palmer, Vernon Hills, IL, USA) at a flow rate of 20 mL/min. Test solutions were pumped from three 4-L aerated reservoirs containing either kairomone, alarm cue, or MHW, and the outlet from all chambers fed into a waste tank. The exchange cycle for the pump was set to a 5-min refresh phase (water flowed through the chambers) and a 25-min measurement phase (water stopped within the chamber) and the entire experiment lasted for 270 min (including acclimation). The 5-min refresh phase was used to prevent oxygen levels from falling to hypoxic conditions between readings and the 25-min measurement phase used to record the oxygen drop associated with fish respiration.

To more readily access the experimental setup, a black cloth was used in place of cardboard as a barrier between the chambers and the rest of the incubator to prevent external stimuli from disrupting the fish. During the 150-min acclimation period, all chambers received a steady flow of untreated MHW.

Following this period, chambers were switched to the corresponding predation cue (K or AC) at the beginning of trials. At the end of the experiment, fish were euthanized using MS-222 immersion, sexed, measured for length (snout to fork) to the nearest 0.1cm, and wet weight taken to the nearest 0.1g. Dry weight was also determined by placing fish in a drying oven (ThermoScientific, Waltham, MA,USA) at 60°C for 24 h. Desiccated fish were weighed to the nearest 0.00001g using a Sartorius CP225D balance(Sartorius AG, Göttingen, Germany). A total of seventy-eight (26 per treatment) mature *G. affinis* (2.6cm-3.6cm) were exposed to treatments.

Temperature-compensated oxygen consumption was recorded (in O_2 mg/L) continuously in each respirometer using infrared robust oxygen probes connected to a Firesting O2 Meter (PyroScience, Aachen, Germany). Metabolic rate in the presence and absence of predation cues was obtained from the decline in oxygen during measurement phases and corrected for background oxygen loss and chamber size.

Influence of Predation Cues on Copper Sensitivity

96-h toxicity tests were used to determine the influence of predation cues on sensitivity of *G. affinis* to copper. Paired bioassays (Fig. 2) were conducted by exposing adult fish to copper chloride alone or in the presence of a predation cue (K or AC). General bioassay methods followed those described by ASTM (1993). Treatments included a control and three copper concentrations with MHW as the diluent. Copper concentrations for alarm cue bioassays (n=5) were 0, 0.25, 0.5, and 1.0 mg Cu/L, while two sets of paired bioassays with kairomone were conducted using lower copper concentrations of 0, 0.125, 0.25, and 0.50 mg Cu/L (n=5) and higher copper concentrations of 0, 0.5, 0.75, and 1.0 mg Cu/L (n=5). Copper treatments were derived from a stock solution prepared with anhydrous copper chloride ($CuCl_2$, Fisher Scientific, Fairlawn, NJ, USA) in deionized water. Treatments containing alarm cue were prepared by adding AC to the test solutions at a concentration of 20 mL AC/L MHW and kairomone bioassays were prepared by using the predator-conditioned water as diluent. Test chambers consisted of 3-L round glass aquaria with two replicates of 10 fish for each treatment. Test chambers were also checked daily for the presence of dead fish as determined by failure to respond to gentle prodding and 75% of each test solution was renewed with fresh solution. A total of 15 paired bioassays were completed.

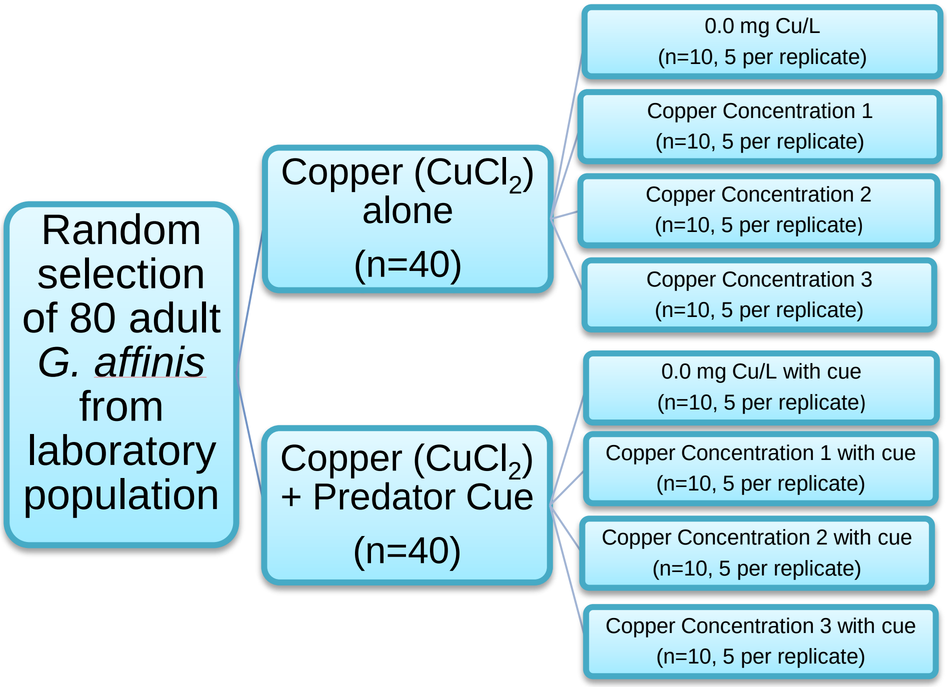

Figure 2. Schematic of paired bioassay toxicity test. Each pair was replicated five times per copper concentration/predatory regimen for a total of 15 tests.

Water Chemistry

During each 96-h experiment, water quality was checked every 24 h. pH was measured with a YSI 4010-2 multipoint meter (Xylem Inc, Yellow Springs, OH, USA), dissolved oxygen and temperature were measured with a YSI model 5000 O_2 meter, and conductivity using a YSI model 3100 Meter. Initial and final alkalinity and hardness were also measured via titration using standard solutions (HACH, Louisville, CO, USA) at the control and highest copper concentrations. Additionally, water samples were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) using an 820-MS ICP Mass Spectrometer (Bruker Co., Billerica, MA, USA) for total copper and, in the high copper concentration kairomone bioassays, for total dissolved copper. Samples for total copper analysis were collected in polyurethane tubes and acidified to pH 2 for holding while samples for total dissolved copper were first vacuum filtered (filter: 0.45 μm pore size, 50 mL diameter, Nalgene, Rochester, NY, USA) and then acidified to pH 2.

Statistical Analysis

All statistical tests were performed at $\alpha=0.05$ in Minitab 17 (Minitab Inc, State College, PA, USA) and figures graphed using SigmaPlot 11 (SysStat Software, San Jose, CA, USA). To investigate if fish were more closely grouping in the presence of alarm cue, an index of cohesion was calculated (Vanzwoll, 2010). The index consisted of recording the most number of fish in a single grid square in each time lapse photo. From these data, a cohesion value was produced by averaging the values recorded for every 2min time period (n=4). Vertical distribution along three levels of water depth were also recorded, but fish across all treatments almost exclusively made use of the lower portion of the aquaria. As such, spatial distribution along the water column could not be analyzed. Index of cohesion values, however, were successfully analyzed using a repeated measures two-way analysis of variance (ANOVA).

Metabolic rates were determined by taking the absolute oxygen consumption of *G. affinis* (n=24 per treatment) exposed to a MHW control, K , or AC (20ml/L). A general linear model (GLM) was conducted using oxygen consumption (mg O_2/min) as the response variable, treatment as the main factor, and dry mass in grams as the covariate. This method has been used successfully as an alternative to mass-specific metabolic rate analysis as it does not assume a linear scaling relationship between oxygen consumption and mass (Rowe, 1998).

Percent mortality data from the toxicity tests were collected at 96 h and compared between treatments. Data were arcsine square-root transformed and then analyzed using a two-way analysis of variance (ANOVA) to determine if the presence of predation cues had an effect on survival.

Results

Water Quality

Dissolved oxygen in $CuCl_2$ treatments alone ranged from 6.11 - 9.06 mg O_2/L while treatments with CuC_{l2} and predation cues ranged from 5.76 - 8.80 mg O_2/L. $CuCl_2$ treatments also had similar ranges of temperature, pH, conductivity, alkalinity, and hardness as compared to treatments with predation cues. Temperature ranged from 18 - 23°C without predation cues and 18.22 - 23.41°C with predation cues. pH values ranged from 6.91 - 8.27 in $CuCl_2$ alone and 6.53 - 8.22 in diluent containing cues. Conductivity ranged 272.20 - 389.80 μS/cm in copper treatments alone and 286.10 - 391.10 μS/cm in chambers with predation cues. $CuCl_2$ treatments had a total alkalinity range from 58 - 74 mg/L $CaCO_3$ and a total hardness range from 78 - 200 mg/L $CaCO_3$. Treatments which included predation cues in addition to copper ranged in total alkalinity from 62-84 mg/L $CaCO_3$ and in total hardness from 80 - 202 mg/L $CaCO_3$. Total copper values and dissolved copper ranged from 1-30% nominal copper concentrations, though outliers were noted.

Effect of Alarm Cue Presence on Behavior

The index of cohesion was not significantly different between *G. affinis* exposed to alarm cue than those exposed to a deionized water (p=0.729) (Fig. 3). There was no interaction between time and treatment (p=0.509); however the index of cohesion was significantly different over time (p=0.046). A significant difference was noted within alarm cue exposed groups between the post-stimulus 2 interval and the post-stimulus 3 interval (p=0.007) but no differences were noted between fish exposed to alarm cue or deionized water within post-interval 2 (p=0.350) or within post-interval 3 (p=0.592).

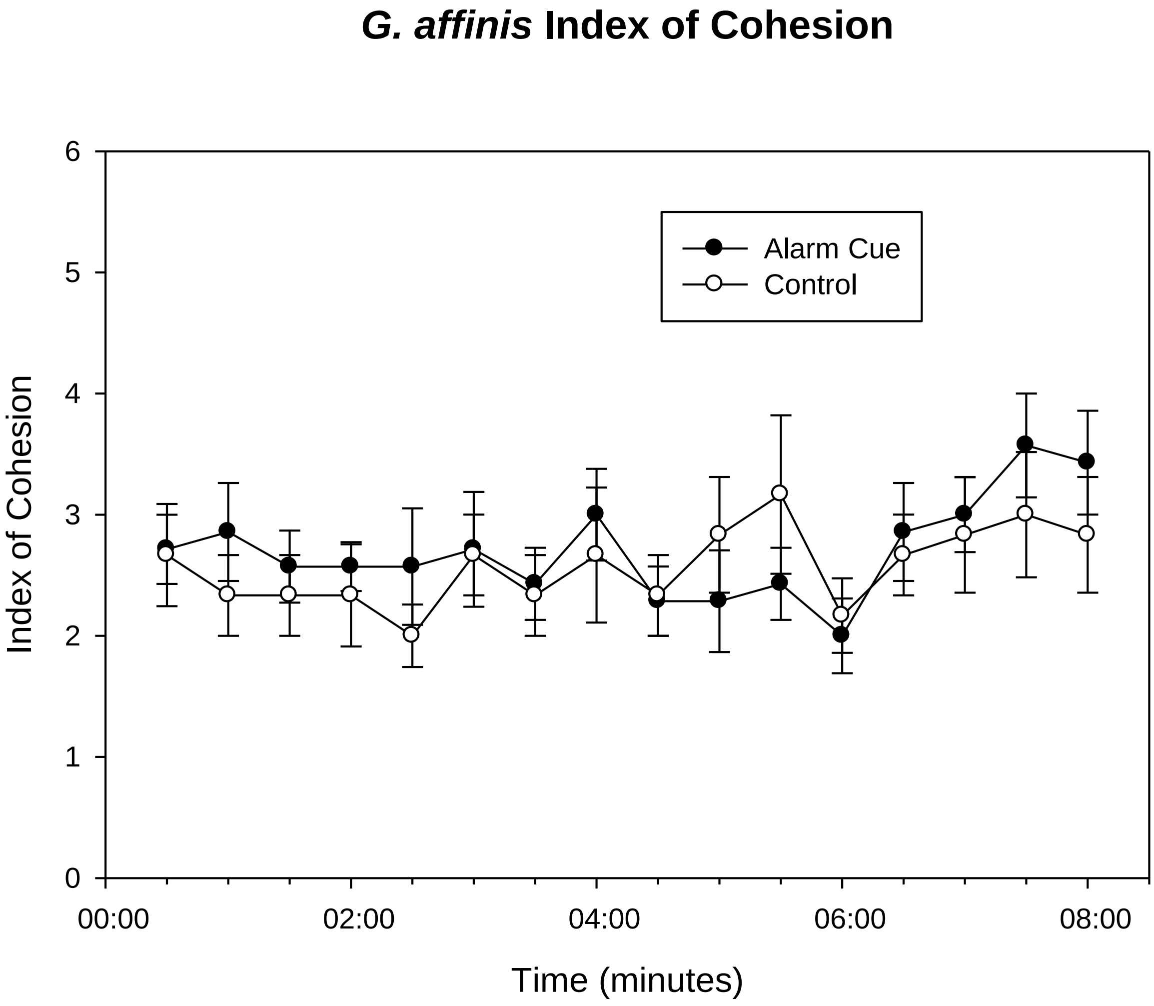

Figure 3. Influence of alarm cue on index of cohesion of *G. affinis* maximum number of fish in a 5cmx5cm plot when presented with stimulus (alarm cue or control) at 2 minutes. Error bars represent 1 standard error.

Effect of Predatory Cues on Copper Chloride Sensitivity

Dose had a significant effect on survival (p<0.001) with decreased survival as copper concentration increased. The presence of AC had no effect on survival (p=0.647) and there was no interaction between the two factors (p=0.886) (Fig. 4).

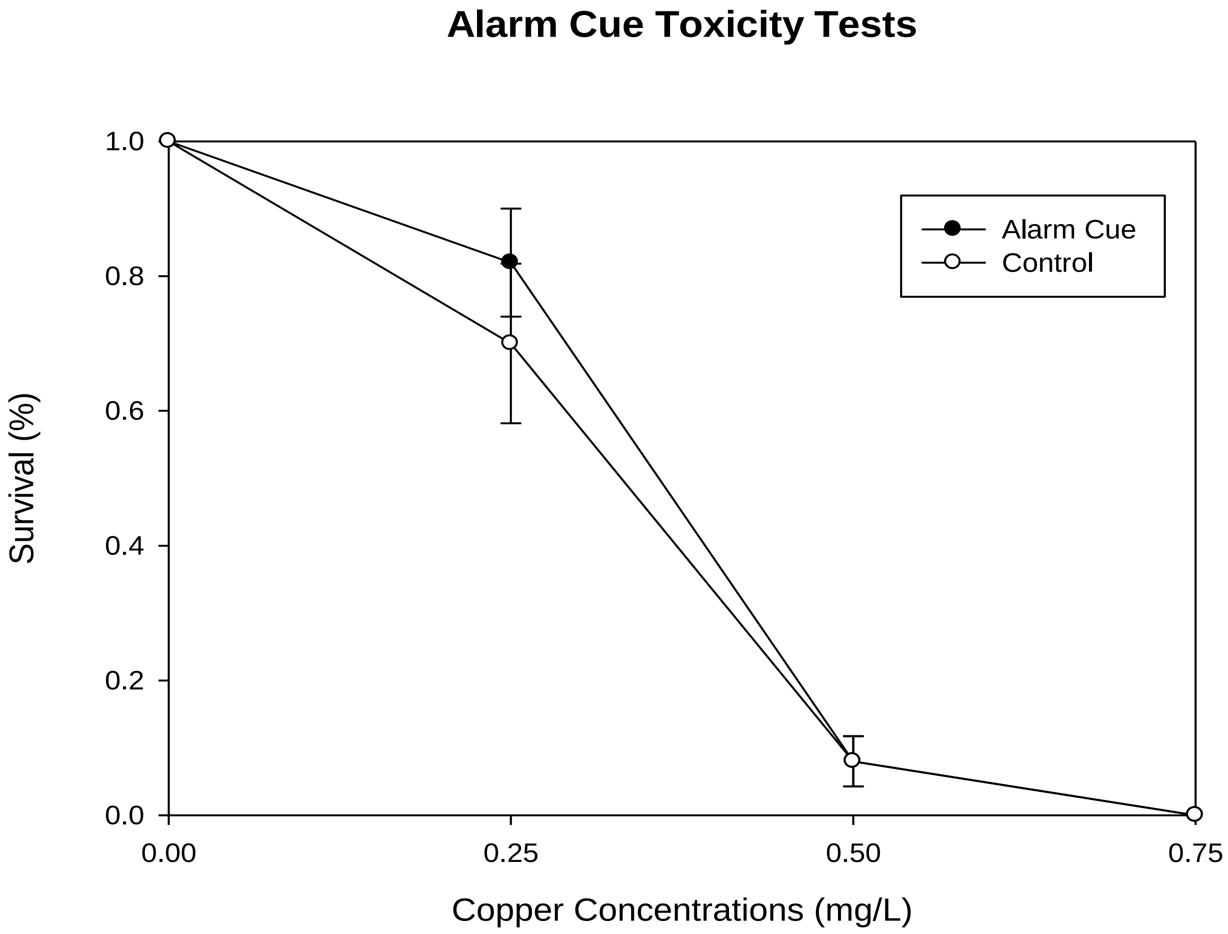

Figure 4. Average survival (n= 5 bioassays) at 96 h for *G. affinis* in the presence of alarm cue (20ml/L) and copper chloride or to copper chloride alone (control). Error bars represent 1 standard error.

Kairomone bioassays were separated into two sets based on copper concentration. Set 1 consisted of 5 bioassays with concentration ranging from 0.00 mg Cu/L - 0.50 mg Cu/L and Set 2 consisted of 5 bioassays with concentration ranging from 0.00 mg Cu/L - 1.00 mg Cu/L. In Kairomone Set 1, the predation cue had no effect on fish survival (Fig. 5, p=0.061) and there was no significant interaction between copper concentration or cue presence (p=0.069). As expected, there was a significant survival response to the copper concentrations (p<0.001). It is interesting to note that while no difference was apparent between treatments across concentrations, survival was significantly increased in the presence of kairomone at a nominal concentration of 0.050mg Cu/L (p=0.005).

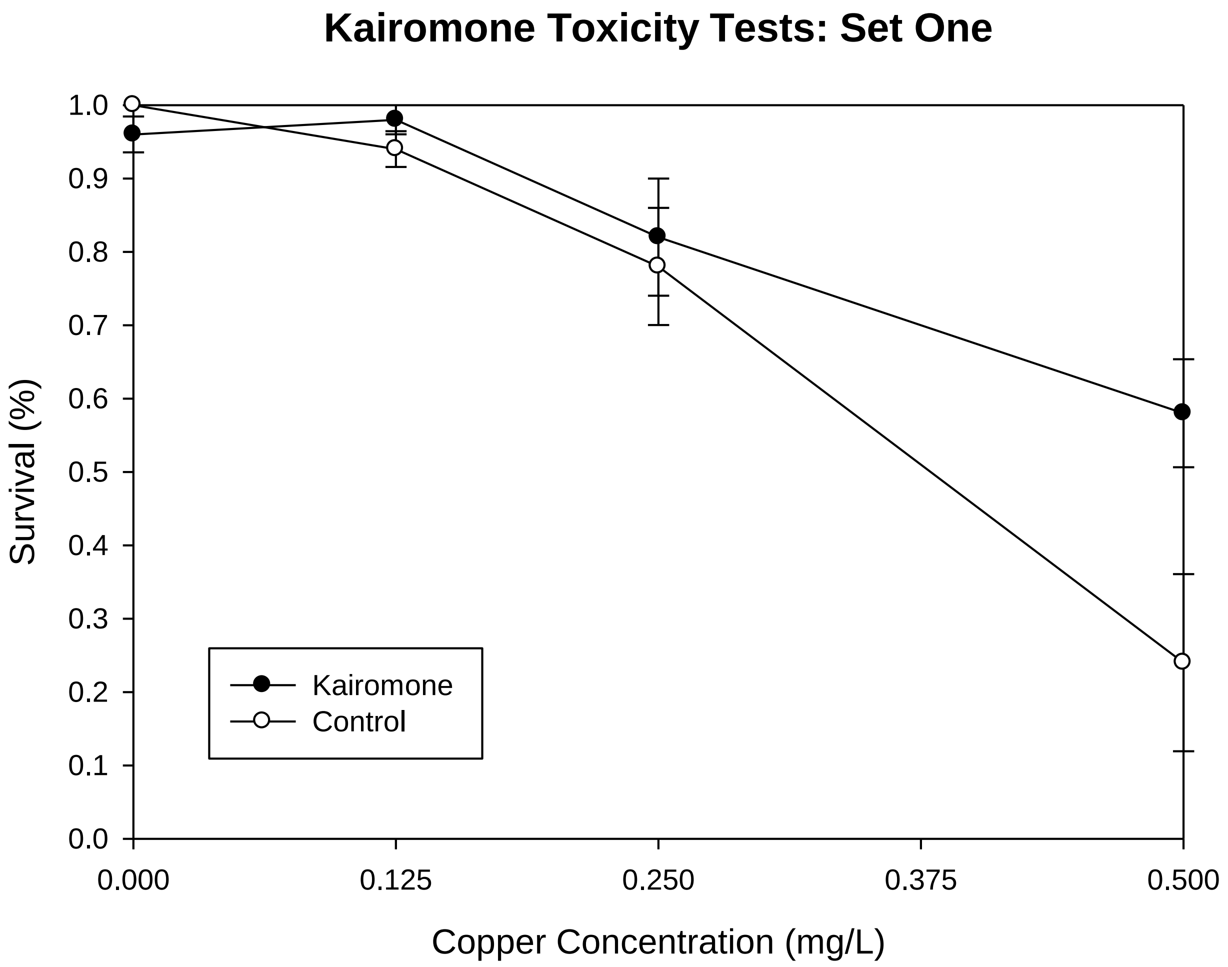

Figure 5. Average survival (n=5 bioassays) at 96 h for *G. affinis* in the presence of bluegill kairomone and copper chloride or to copper chloride alone (control). Error bars represent 1 standard error.

In Kairomone Set 2, predation cues had a significant effect between treatments (p=0.001) and a significant dose response on mortality in the tested copper concentration (Fig. 6, p<0.001). In addition, concentration had with an interaction effect with survival (p=0.007).

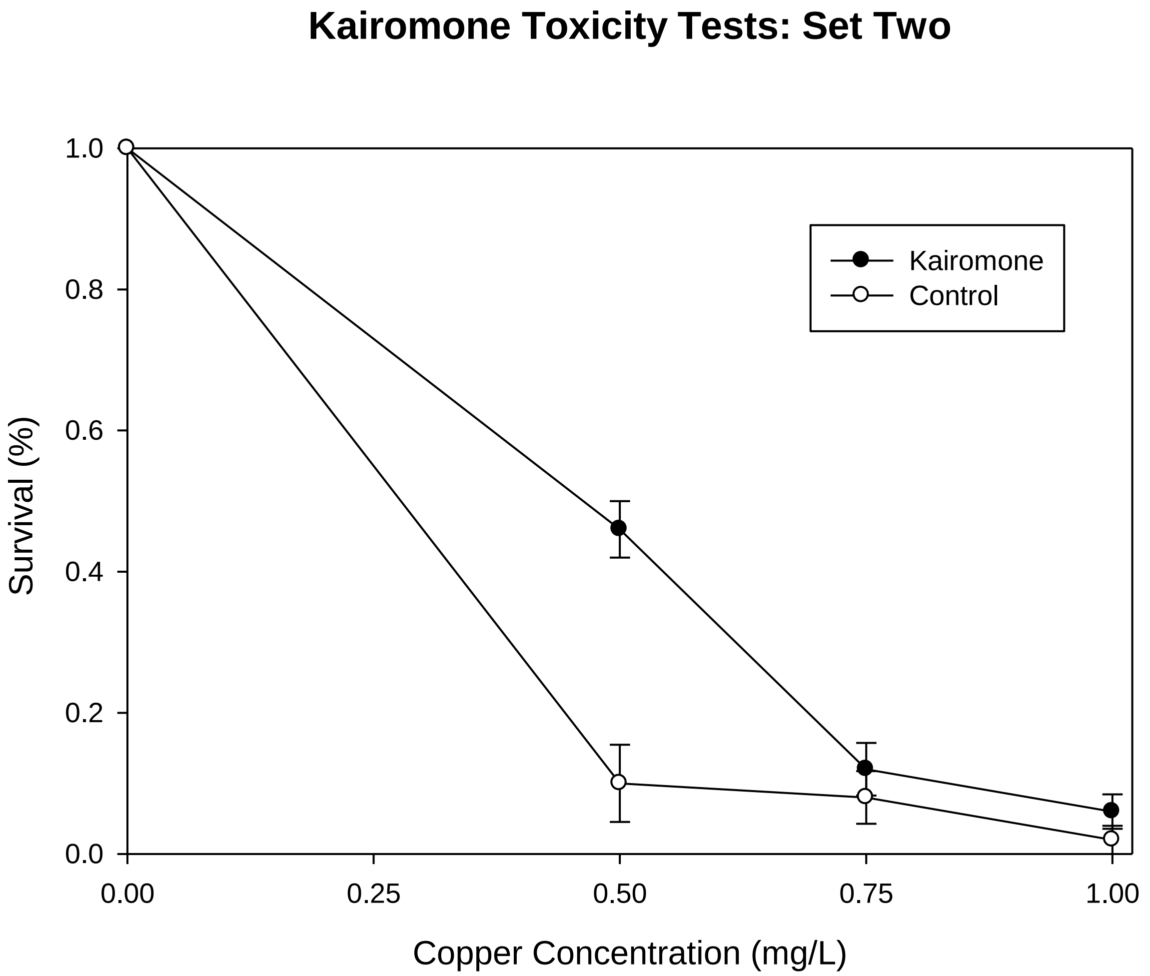

Figure 6. Average survival (n=5 bioassays) at 96 h for *G. affinis* in the presence of bluegill kairomone and copper chloride or to copper chloride alone (control). Error bars represent 1 standard error

<u>Effect of Predatory Cues on *Gambusia* Metabolic Rate</u>

Mass had a significant effect on oxygen consumption ($p<0.001$). Neither the presence of conspecific alarm cue or the presence kairomone had a significant effect on *Gambusia* oxygen consumption (Fig. 7, $p=0.114$).

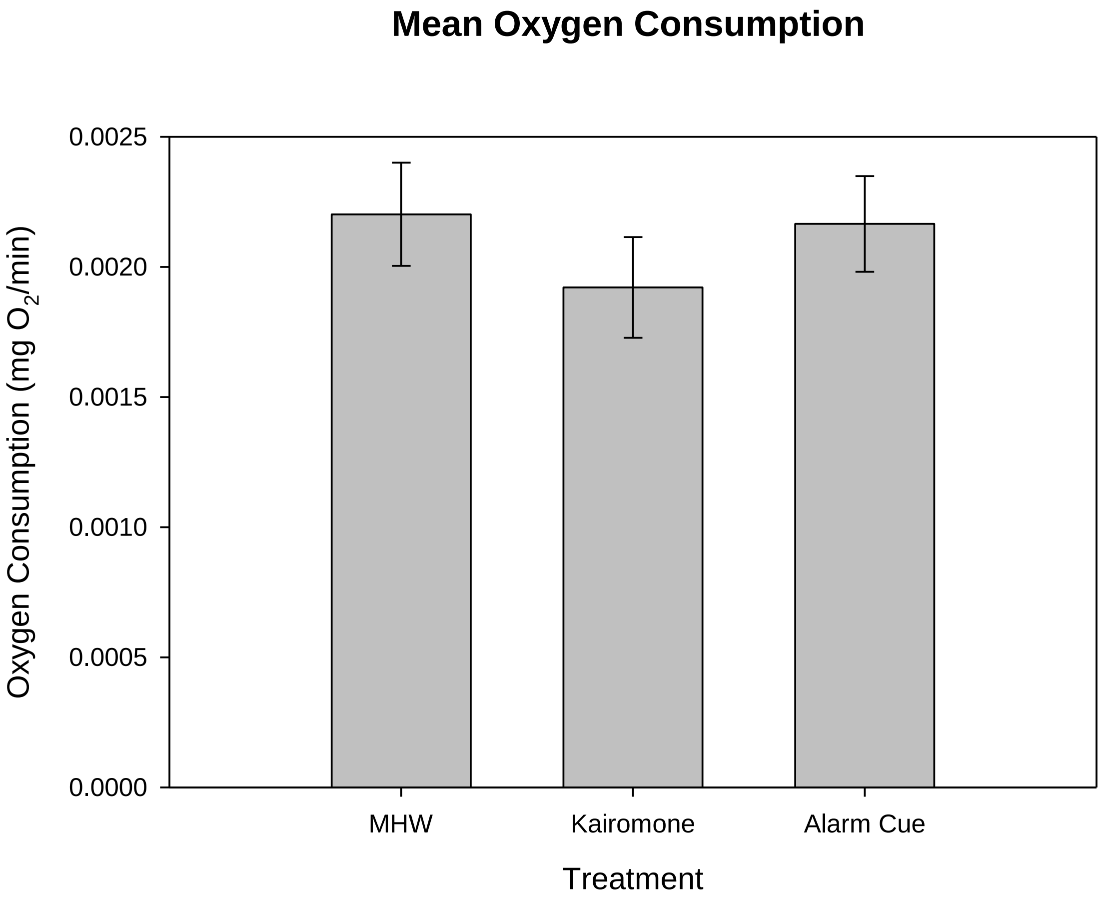

Figure 7. Mean oxygen consumption in G. affinis exposed to bluegill kairomone, alarm cue (20ml/L) or moderately hard water (control). Error bars represent 1 standard error.

Discussion

Effects of Alarm Cue on *Gambusia* Cohesion

We found no difference in the index of cohesion (grouping behavior) of *Gambusia* in the presence of conspecific alarm cue when compared to controls, although there was a temporal effect on cohesion in fish exposed to alarm cue. Previous studies have found that both *G. affinis* and the closely related *G. holbrooki* exhibit predator avoidance behaviors when presented with alarm cue (Bryant, 1986; Garcia et al. 1992; Reed, 1969; Vanzwoll, 2010; Ward, 2011). Our methods varied from Vanzwoll (2010), who conducted behavioral trials using *G. holbrooki*, in two ways. Firstly, we made use of *G. affinis* vertical distribution by using Garcia et al. (1992) methods in case the index of cohesion was not an appropriate behavioral indicator. However, fish were consistently located toward the bottom of the aquaria regardless of treatment and these data were unable to be analyzed. Secondly, we made use of three post-stimulus observation phases instead of four (-2 min). Increasing the trial period may have resulted in the observation of predator avoidance behaviors, but responses are often known to occur upon cue detection. Preliminary diffusion experiments indicated complete dispersal of the cue in the aquaria within one minute of stimuli introduction, suggesting fish should have detected cue within the timeframe of the experiment.

Similarly to work by Bryant (1986) and Reed (1969), only mature fish were used to make and test alarm cue. This would reduce or eliminate any potential age/developmental differences which may have affected responses (Mitchell and McCormick, 2013). Additionally, a school of six individuals was used for each exposure treatment. This size of school falls into that used in treatments by Reed (1969). The lack of response to alarm cues may then have stemmed from collection procedures. Garcia et al. (1992) examined whether the presence of a sacrificed animal, homogenized in 20 mL of water, would cause a behavioral response. Bryant (1986) used macerated skin from 12 *G. affinis* homogenized in 150 ml water. Both alarm cue collection methods produced predator avoidance behaviors. Our collection methods differ from these previously used procedures. Homogenized skin (0.1 cm^2 skin/mL) was used to make alarm cue for *G. affinis* toxicity testing, and a sacrificed conspecific was rinsed using deionized water for behavioral assays. Fish may not respond to cues collected in this manner. Vanzwoll (2010) successfully used the index of cohesion and alarm cues rinsed from a sacrificed conspecific to produce predator avoidance behavior in *G. holbrooki*. However, these methods have not been used previously in *G. affinis.* The lack of response to cue we noted may have stemmed from these differences from past studies, but a biological reasoning cannot be completely discluded.

Gambusia may be found in habitats that differ in terms of resource availability and predation pressure. Predator avoidance behaviors are phenotypically plastic, and have been found to vary between populations in fish. Different populations of the same species may make use of different anti-predator strategies (Foster, 1999; Templeton and Shiner, 2004). In *Gambusia,* predation is a selective force on body morphology (Laggerhans et al. 2004) and may affect behavioral strategy as well. While vertical distribution and schooling behavior have been used as a successful indicator of alarm cue detection in prior work (Garcia et al. 1992, Reed 1966), the fish used in this study may exhibit a different behavioral response to predator threat that was not characterized in this work. Alternatively, *G. affinis* may respond differently to varying concentrations of alarm cues.

Work with *G. affinis* has made use of a number of differing alarm cue concentrations, but to our knowledge, none have characterized whether they respond along a concentration gradient (Mirza and Chivers, 2003). The method which cue was collected or the concentration tested may not cause fish to respond behaviorally. Future behavioral testing in *G. affinis* should investigate freezing behavior, another behavioral response noted in *G. affinis* (Bryant, 1986), as well as determine if behavioral responses are reduced or eliminated at different concentrations of alarm cue.

Effect of Predatory Cues on *Gambusia* Sensitivity to Copper Chloride and Metabolic Rate

Concentrations below 0.50 mg Cu/L did not cause significant lethality (~20%) in *Gambusia* whereas concentrations above 0.50 mg Cu/L resulted in a large die-off (>80%). In *Gambusia*, mortality varied at 0.50 mg Cu/L between treatment assays. At similar levels of hardness to the present work, previous studies have reported median lethal concentrations for copper (LC_{50}) ranging from 0.38 mg Cu/L - 0.72 mg Cu/L (Suter and Tsao, 1996). *G. affinis* sister species', *G. holbrooki*, have a comparable LC_{50} value of 0.78 mg Cu/L (Saez et al. 2013). Studies have demonstrated that *Gambusia* copper sensitivity is dependent on life-history, population structure, and abiotic conditions. Fish can survive in copper concentrations ranging from 0.20 mg Cu/L to 5.0 mg/L (Joshi and Rege, 1980; Kallanagoudar and Patil, 1997; Sharma et al. 2007).

Alarm cue did not affect copper tolerance in *G. affinis*. This outcome may be associated with the lack of response to alarm cue noted in the behavioral assay and is further supported by the metabolic rate data. When corrected for mass, *Gambusia* exposed to alarm cue showed no significant changes in oxygen consumption as compared to control fish. Copper has been shown to impair olfaction during the developmental stages as well as in adult fish (Pyle and Mirza, 2007). For example, Colorado pikeminnow (*Ptychoheilus lucius*) exposed to copper concentrations ranging from 10 - 12 µg/L exhibited a dose-dependent reduction in response to alarm cues (Beyers and Farmer, 2001). *Gambusia*'s ability to respond to alarm cue in toxicological tests may have been compromised, however fish showed no differences in behavior between the untreated controls and those exposed to copper. Fish were not exposed to copper before oxygen consumption was recorded so their ability to detect predation cues should have been unaffected.

The presence of bluegill kairomone also had no effect on survival of *Gambusia* at copper concentrations ranging from 0.25 - 0.50 mg Cu/L. However, there was an increase in *Gambusia* survival at the highest concentration of copper (0.50 mg Cu/L). To further investigate this decrease in copper sensitivity, a secondary set of bioassays were conducted using copper ranging from 0.50 - 1.0 mg Cu/L. As for the first set of bioassays, the presence of kairomone appeared to increase *Gambusia* survival. This was most pronounced at 0.50mg Cu/L.

A number of explanations have been proposed for this type of interaction. Previous work using pesticides shows that predatory cues may increase, decrease, or have no effect on toxicity of the chemical (Relyea, 2003, 2012; Robison, 2010). For example, when exposed to malathion, tadpoles (*Euflictus cyanophlystis*) did not experience an increase in sensitivity to the pesticide (Giri et al. 2012). Additionally, tadpoles of different species were affected by the presence of carbaryl in combination with predator kairomones synergistically, or an interaction did not occur at all (Relyea and Mills, 2001). Our results suggest that conspecific alarm cues do not alter *Gambusia* tolerance to copper but predator kairomones may enhance tolerance at certain concentrations. The increased survival noted at 0.50 mg Cu/L could be explained by behavioral changes in *Gambusia.* Specifically, *Gambusia* may exhibit reduced movement in the presence of kairomone as suggested previously. This would in turn decrease ventilation rate and the amount of copper uptake across the gills (McGear et al. 2000).

While *Gambusia* have been shown to recognize and react to predator kairomones behaviorally (Reed, 1969; Smith and Belk, 2001). This behavioral explanation is not supported in the current work. When exposed to bluegill kairomone *Gambusia* did not reduce oxygen consumption as would be expected with a reduction in movement. In fact, no response was noted. The lack of changes in oxygen consumption in response to predatory cues may be due to the environment from which the *Gambusia* originated. Fish from high predation sites, as opposed to those with little to no predator presence, have been to shown to have overall decreased stress response to predators (Brown et al. 2005). From the presence of kairomone, *Gambusia* are able to discern predator size, state of hunger, and diet (Smith and Belk, 2001). It is possible that the *Gambusia* from the current site may have an already dampened rate of oxygen consumption and show no changes in kairomone presence. However, it is possible they may simply not respond. Further investigation into oxygen consumption in the species between populations with varying predator densities may be beneficial.

Decreased toxicity associated with kairomone presence in some contaminants has been attributed to binding with organic material found in predator-treated water. This reaction decreases the concentration of contaminant available for uptake by organisms (Qin et al. 2011). In invertebrates, this mechanism was suggested to explain reduced toxicity of silver nanoparticles to *Daphnia carinata* (Qin et al. 2015). In fish, the presence of organic material was a potential mechanism for why fathead minnows expressed no change in cadmium tolerance (Robison, 2010). Copper is toxic as it binds readily to fish gills in its free ion form. At 0.50 mg Cu/L, the presence of kairomone may have created an increase of organically bound copper, thus reducing the bioavailable concentration able to cross over the gills.

The binding of copper to organic material, and its antagonistic effect on toxicity, are dependent on water quality and dissolved metal in the environment (Paquin et al. 2002). A large decrease in mortality was not noted in high kairomone treated copper concentrations. At these concentrations though some copper may still be unavailable to cross the gills due to binding with organic compounds, the number of free ions may still be present in concentrations which affect the physiology of the fish. As such, the antagonistic effected noted at 0.50 mg Cu/L would no longer be present as noted at 0.75 mg Cu/L and 1.0 mg Cu/L. To support this, the total copper concentrations should be notably larger than the dissolved copper concentrations in kairomone bioassays. This was not the case in our study, as our data shows a relatively similar range (~20-25% of the nominal concentration) of copper, regardless of the presence of or absence of kairomone.

Conclusions

Exposure of *Gambusia* to alarm cue resulted in no change in behavior, metabolic rate, or sensitivity to copper. The alarm cue in *Gambusia* has not been characterized but has been confirmed through numerous behavioral assays (Pyke, 2005). A complete lack of response to alarm cue has been noted in *Gambusia* in early studies (Reed, 1969), though whether these were *G. affinis* or *G. holbrooki* is unknown. Potentially, *Gambusia* from the current population may lack a response to alarm cue, or only respond consistently with 'fight or flight' to a certain concentration of alarm cue. Factors such as life history, genotype, and nearby conspecific behaviors are known to affect fish behavior, and may play a role in the lack of response noted here (Cote et al. 2010, Herczeg et al. 2016). Additionally, the lack of response may stem from the way in which alarm cues were prepared.

Exposure of *Gambusia* to kairomone did not alter oxygen consumption and had no effect on survival at copper concentrations ranging from 0.25 - 0.50 mg Cu/L. *Gambusia* survival increased in copper exposures ranging from 0.50 - 1.0 mg Cu/L. This was most pronounced at 0.50 mg Cu/L. Our results suggest this is dependent on copper concentration. Concentrations which are too low will not affect fish, while concentrations that are too high will result in death. Kairomone effect on *Gambusia* survival at 0.50 mg Cu/L may stem from the bioavailability of copper. While our results are mainly negative, this study adds the body of work which notes the complexities and diverse responses in vertebrates to combined natural and anthropogenic stressors (Relyea, 2003; Robison, 2010).

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APPENDEX

Table 1. Total copper concentration in test chambers compared to nominal value. Numbers in italics beneath measured concentration represent percent deviation from nominal value. (-) represents no sample available.

Paired Bioassays: Total Copper (mg Cu/L)										
	Treatment									
Nominal Concentration	**MHW**					**Alarm Cue**				
0.25		0.307	0.278	0.825	0.301		0.287	0.308	0.268	0.294
		22.82%	*11.17%*	*230.30%*	*20.68%*		*14.83%*	*23.22%*	*7.37%*	*17.64%*
0.5		0.501	0.547	0.523	0.558		0.568	0.582	0.457	0.572
		0.19%	*9.39%*	*4.74%*	*11.75%*		*13.69%*	*16.40%*	*-8.42%*	*14.42%*
0.75		0.899	0.797	0.686	-		0.872	0.792	0.676	0.717
		19.90%	*6.32%*	*-8.53%*	-		*16.31%*	*5.69%*	*-9.85%*	*-4.44%*
Kairomone Set One Bioassays: Total Copper (mg Cu/L)										
	MHW					**Kairomone**				
0	0.095	0	0	-	-	0.001	0	0	-	-
	9.50%	*0*	*0*	-	-	*1.68%*	*0*	*0*	-	-
0.125	0.108	0.097	0.085	0.092	0.081	0.105	0.129	0.088	0.0815	0.099
	-12.82%	*-22.21%*	*-31.66%*	*-25.79%*	-34.43%	*-15.79%*	*3.63%*	*-29.07%*	*-34.82%*	*-20.41%*
0.25	0.205	0.193	0.181	0.187	0.178	0.211	0.359	0.192	0.172	0.189
	-17.92%	*-22.48%*	*-27.48%*	*-24.98%*	*-28.79%*	*-15.35%*	43.96%	*-22.86%*	-31.08%	*-24.06%*
0.5	0.424	0.43	0.366	0.402	0.403	0.442	0.419	0.396	0.369	0.408
	-15.11%	*-13.85%*	*-26.68%*	*-19.55%*	*-19.22%*	*-11.46%*	*-16.10%*	*-26.68%*	*-26.09%*	*-18.38%*

Table 2. Total copper and dissolved copper concentrations in test chambers compared to nominal value. Numbers in italics beneath measured concentration represent percent deviation from nominal value. (-) represents no sample available.

Kairomone Set Two Bioassays										
Total Copper (mg Cu/L)										
	Treatment									
Nominal Concentration	**MHW**					**Kairomone**				
0	0.15	-	-	-	-	0.016	-	-	-	-
	15.15%	-	-	-	-	*1.6%*	-	-	-	
0.5	0.389	0.397	0.408	0.670	0.602	0.405	0.372	0.414	0.644	0.623
	-22.01%	*-20.6%*	*-18.22%*	*34.07%*	*20.55%*	*-18.99%*	*-25.43%*	*-17.08%*	*28.92%*	*24.77%*
0.75	0.623	0.580	0.513	1.64	0.96	0.641	0.61	0.523	1.142	0.941
	-16.81%	*-22.64%*	-31.56	119.79%	*28.18%*	*-14.41*	*-27%*	*-30.24%*	52.35%	*25.51%*
1.0	0.875	0.778	0667	1.173	1.16	0.667	0.782	0.313	1.41	1.45
	-12.41%	*-22.16%*	*-33.21%*	*17.35%*	*16.17%*	-33.2	*-21.77%*	*-68.6%*	*40.9%*	*45.28%*
Dissolved Copper (mg Cu/L)										
0.5	0.385	0.357	0.411	-	0.492	0.402	0.381	0.613	-	0.518
	-22.83%	*-28.46%*	*-17.69%*	-	*-1.49%*	*-19.59%*	*-23.67%*	*22.75%*	-	*3.69%*
0.75	-	-	-	-	0.774	-	-	-	-	0.749
	-	-	-	-	*3.25%*	-	-	-	-	*-0.01*
1.0	-	-	-	1.08	-	-	-	-	1.17	-
	-	-	-	*8.78%*	-	-	-	-	*17.95%*	-

VITA

MELISSA NICHOLE CAMPBELL

Education:

Public Schools: Bethel, Bethel Township, Ohio 2008

B.S. Zoology, *cum laude*, Ohio State University, Columbus, Ohio 2012, Minor: English

M.S. Biology, East Tennessee State University, Johnson City, Tennessee 2017

Professional Experience:

Graduate Assistant: Science Consult for North Side Elementary, East Tennessee State University; Johnson City, Tennessee 2014-2016

Graduate Teaching Assistant, East Tennessee State University, Johnson City, Tennessee 2016

Awards and Grants:

Scholarship: East Tennessee State University Thesis/Dissertation Scholarship, 2017

Award: Graduate Master's Poster Presentations Second Place; Appalachian Student Research Forum, EFFECTS OF PREDATION CUES ON METAL TOXICITY IN *GAMBUSIA AFFINIS*, 2016

Grant: Dr. Denise Pav Research/Scholarship Award, East Tennessee State University 2016

Activities

Biological Outreach North Side Elementary, East Tennessee State University, 2014-2016

Regional Science Fair Judge, East Tennessee State University, 2016 & 2017

Memberships: American Association for the Advancement of Science

Publication

Campbell, M., and Roberts, J.A. 2015. Influence of ambient temperature on efficacy of signals produced by female *Schizocosa ocreata (Hentz, 1844) (Araneae: Lycosidae)*. Journal of Arachnology, 43 (3): 422-424