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Enemy Exacerbation: Effects of Predator Stress on Sulfate Lethality in Freshwater Amphipods
(*Gammarus minus*)

A thesis
presented to
the faculty of the Department of Biological Sciences
East Tennessee State University
In partial fulfillment
of the requirements for the degree
Master of Science in Biology

by
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August 2017

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Keywords: *Gammarus minus*, sodium sulfate, predator cues, *Gambusia affinis*, kairomone, lethality, respirometry

ABSTRACT

Enemy Exacerbation: Effects of Predator Stress on Sulfate Lethality in Freshwater Amphipods
(*Gammarus minus*)

by

Trevor L. Chapman

Predator cues can influence how aquatic organisms respond to anthropogenic contaminants. This study examined the effects of predator cues on behavior, metabolic rate, and sulfate (as Na₂SO₄) toxicity in amphipods (*Gammarus minus*). Predator cues included alarm cue (macerated conspecifics) and kairomone from mosquitofish (*Gambusia affinis*). Amphipods decreased activity and increased time in refuge when exposed to alarm cue, and increased time in refuge when exposed to kairomone. While median lethal concentrations (96-h LC₅₀) were not influenced by predator cues, analysis of dose response curves indicated that kairomone exposure increased amphipod sensitivity to mid-range concentrations of sulfate (500-1,000 mg/L). Amphipods increased oxygen consumption in response to kairomone but not alarm cue. The influence of predator cues on contaminant lethality can be dependent on the type of cue, and physiological endpoints such as metabolic rate may help explain the basis of observed interactions.

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CHAPTER 1

LITERATURE REVIEW

Project Overview

Environmental degradation leading to decreased biological diversity has been a focus of conservation research for decades (Sinclair et al. 1995; Fahrig 2003). In aquatic systems, toxicological studies have been used to determine how animals respond to sources of anthropogenic stress (Timbrell 1998). While previous studies have demonstrated the impacts of numerous types of human contamination on organisms, most have only focused on the effect that a single stressor has on an organism (Maltby et al. 2000; Relyea 2003). The field of toxicology has begun to incorporate natural stressors (e.g., population density, predation), which allows evaluation of how an organism will respond to anthropogenic stressors in more realistic settings (Relyea and Mills 2001).

Currently, most studies that analyze effects of natural stressors on contaminant lethality have focused on pesticide contaminants. For example, in a study by Relyea (2005), six species of frog tadpoles were exposed to varying concentrations of glyphosate and glyphosate combined with predator cues. Results suggested that glyphosate lethality increases at median concentrations (neither 100% survival nor total mortality) in the presence of predator stress. Similarly, Jones et al. (2011) demonstrated that glyphosate lethality increased at higher densities in tadpole populations. The effect of multiple stressors is not restricted to vertebrates. When *Daphnia* are exposed to both carbaryl and parasite stress, mortality is significantly higher than when they are exposed to either stressor individually (Coors and De Meester 2008). While there is research indicating how multiple stressors affect contaminant lethality, studies that examine

the underlying causes of the interactions are lacking. Relyea and Mills (2001) hypothesized that the combined stressors phenomenon could be due to an interaction between two stressors, wherein the general stress caused by the predator presence exacerbates the toxic effects of the contaminant.

The General Adaptation Syndrome (G.A.S.) describes how physiological responses to stressors manifest in vertebrate organisms (Selye 1985). In this context, a stressor is defined as some agent that produces a nonspecific response by triggering pathways in the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis (Veissier and Boissy 2007). However, stressors can also induce specific stress responses, as is often seen in later stages of viral or pathogen infection (Selye 1985). The G.A.S. progresses in three stages: (1) the “alarm reaction”, (2) the “stage of resistance”, and (3) the “stage of exhaustion” (Selye 1985). In the alarm reaction, an organism responds to stressors with internal changes, such as metabolism, hormone levels, and blood pressure. This is also referred to as stress, or the stress response. The stage of resistance, or adaptation, describes the subsequent opportunity for the body to counteract the physiological stress response and return to homeostasis. This could include balancing hormones or returning to a resting metabolic rate. If the adaptation is not achieved and the chain of reactions does not stop, then the organism eventually reaches the stage of exhaustion, or death (Selye 1965).

Although the G.A.S. is mostly referenced for vertebrates, the general sequence of events are often observed in invertebrates. For example, neurohormones such as octopamine are released during fight or flight response in many insects and cause physiological responses similar to norepinephrine in vertebrates (Adamo et al. 1995). While the G.A.S. describes a generalized response to stress, it is hypothesized that exposure to multiple stressors can accelerate or inhibit

progression through each stage (Patton 1970). In this study, the hypothesis is that additional stressors can induce further stress on the organism, influencing their capacity to cope with the effects of a chemical stressor by altering the intensity of the physiological response. This could mean that energy is diverted from mechanisms that would normally work to resist the chemical stressor so that the sensitivity to the stressor increases. For example, while an organism may be able to tolerate moderately high levels of thermal stress and increased metabolic rates, an additional stressor that also impacts metabolic rates could influence tolerance of thermal stress. Therefore it is important to analyze the effects of anthropogenic stressors in the presence of stressors that an organism might encounter in its natural habitat.

The pathways described by the G.A.S. provide evidence for several physiological changes that can be used as indicators of stress. One response that has been well studied is metabolic rate (Barton and Schreck 1987;Anestis et al. 2007;Brotman et al. 2007). While stress does not always appear to directly cause an increase in oxygen consumption initially, the pathways initiated during an alarm response contribute to physiological alterations that eventually affect respiration (Brown 2003). For example, a stressor that signals danger can cause increased arousal or alertness. In order for the body to remain alert and prepared for a potentially dangerous encounter, oxygen is directed to the musculature and also is used for increased ATP synthesis, both of which can lead to increased respiration (Chrousos 1998). Other studies have shown that predator pressure can increase respiratory rates in prey organisms (Biebuyck and Phil 1990;Sapolsky et al. 2000;Bell et al. 2010).

To date, the majority of studies involving natural and anthropogenic stressors have focused on predator cues combined with different pesticides. However, a study (Robison et al. Manuscript in Review) examined the effects of combined predator stress and chemical stressor in

fathead minnow larvae (*Pimephales promelas*) and water flea neonates (*Daphnia pulex*). Results indicated that while alarm cue caused a decreased sensitivity to sodium chloride in minnow larvae, predator cue alone did not. These results were further supported by oxygen consumption trials which indicated increased oxygen consumption in response to alarm cue. However, when larvae were exposed to cadmium chloride instead of sodium chloride, the predator cues had no effect. In the same study, daphnids exhibited a differential response to sodium chloride combined with multiple predator cues, wherein some of the predator cues caused a decrease in sensitivity with decreased rates of oxygen consumption while others did not. These results suggest that the effect of combined stressors can rely heavily on the test organism, chemical contaminant of interest, and type of predator stress.

This study analyzed the effects of predator cues on sodium sulfate lethality in a species of freshwater amphipod (*Gammarus minus*). Lethality trials were conducted with and without predator cues to determine whether the cues affected sensitivity to sulfate. In order to address the question as to whether the effect of combined stressors is due to a general stress response, oxygen consumption trials were also conducted.

Model Organism

A species of freshwater amphipod (*Gammarus minus*) was used as a model organism in this study. *Gammarus* amphipods are globally distributed and commonly used as model organisms in exotoxicology (Costa et al. 2009). The *Gammarus* genus is speciose (over 200 species) and is highly diverse in terms of community function as shredders, predators, grazers, and detritivores (Kelly et al. 2002).

The organisms used in this study were collected from a spring in Elliston, Virginia (Montgomery County, 37.21417°, 80.23667°). The stream was spring fed, and maintained a temperature of 13 to 15° C year round. Potential predators observed in the stream included freshwater crayfish (species not identified), two-lined salamander larvae (*Eurycea cirrigera*), and one darter minnow (species not identified).

Anthropogenic Stressor

Although most research into the effects of predator cues on toxicity have used pesticides, sodium sulfate was used in this study. Sulfate is an example of a major ion that is ordinarily harmless at low concentrations relative to other contaminants such as pesticides, but can be detrimental to aquatic organisms at high concentrations (Chapman et al. 2000; Kennedy et al. 2005; Soucek and Kennedy 2005). For example, a study by Elphick et al. (2011) exposed numerous aquatic invertebrates and vertebrates to varying sulfate concentrations, and results showed that sensitivity varies among species. There was also a strong effect of water hardness on sulfate sensitivity, which has also been observed with chloride (Soucek et al. 2011). Furthermore, Soucek and Kennedy (2005) demonstrated that even relatively low concentrations of sodium sulfate caused decreased fecundity in the daphnid *Ceriodaphnia dubia*.

Sodium sulfate is one waste product of coal mining and ore refining, which is relevant to the Appalachian region (Birge et al. 1989). Field studies have provided evidence that the effluent from coal processing facilities contains total dissolved solids (TDS) concentrations reaching levels that have been demonstrated to cause harm to aquatic organisms (Chapman et al. 2000; Soucek and Kennedy 2005). Major ion toxicity in the form of TDS is also a byproduct of other sources such as food processing, reverse osmosis, oil and gas refining, agricultural runoff, and groundwater remediation (Ingersoll et al. 1992; Mickley et al. 1996; Pillard et al. 1996; Tietge

et al. 1997; Goodfellow et al. 2000). Currently, major ions are regulated by some state environmental agencies but not federal agencies (USEPA 2016).

Natural Stressor

The natural stressor used in this study is the presence of a predator. In aquatic systems, it is common for organisms to rely on detection of chemical cues from conspecifics or heterospecifics to acquire information about their surroundings (Ferrari et al. 2010). The ability to assess nearby individuals is often used to learn about foraging opportunities, locate conspecifics or mates, and is very prominent in predator-prey interactions of both prey location and predator detection. If a prey species is able to chemically detect a predator, it may be able to alter its behavior to increase its chance of survival (Lima and Dill 1990). This response to predator cues is commonly known as the “fight or flight” response, and it is a component of the G.A.S. However, predator stress is also known to induce neurological and physiological changes in prey organisms (Slos and Stoks 2008; Sanogo et al. 2011). For example, studies have demonstrated that predator stress has a significant effect on the developmental rate of several anuran tadpoles, including wood frogs (*Rana sylvatica*; Relyea and Mills 2001).

There are two means of chemical communication by which prey may assess predator danger: (1) alarm cues and (2) kairomones. Kairomones are substances passively released as a product of metabolism by heterospecific predators that prey can identify (Ferrari et al. 2010). Conversely, alarm cues are chemical signals released by conspecifics that can potentially warn nearby individuals of predation events (Chivers and Smith 1998). Alarm cues have been identified in several species of organisms across many taxa. Isopods, tadpoles, salamanders, and fish are all examples of groups with at least one species that responds to alarm cues with predator avoidance behavior (Rajchard 2006; Brown-Wilusz 2008; Spivey et al. 2015). In Ostariophysian

fish, the alarm substance has been identified as a nitrogen oxide compound, now known as “Schreckstoff”, which is released when skin club cells are ruptured (Reed 1969). Although the substance has not been identified in many species including amphipods, previous studies have demonstrated that chemical cues from injured conspecifics induce a predator avoidance response in amphipods (Wisenden et al. 1999).

In this study, both kairomone and alarm cue were used as natural stressors. The methodology used in previous studies of this type have exposed the model organisms to constant predator stress by means of a caged predator (Relyea and Mills 2001). Incorporating both types of predator cue, rather than just kairomone, provides a more thorough assessment of potential predator stress in a natural environment.

CHAPTER 2

ENEMY EXACERBATION: EFFECTS OF PREDATOR STRESS ON SULFATE LETHALITY IN FRESHWATER AMPHIPODS (*Gammarus minus*)

Introduction

Single species toxicity tests are a commonly used method for assessing the risk that anthropogenic chemicals pose to the environment (Maltby et al. 2000; Relyea 2003). In single species tests, one species is exposed to a single contaminant in a controlled laboratory setting. These tests are often repeated with several model species in order to understand the effects on different types of organisms. These studies can provide valuable information about how different species respond to a single contaminant (e.g., glyphosate), but they lack any elements of community interaction that takes place in a natural environment. Researchers in ecotoxicology have begun to incorporate natural stressors, such as predator cues, into studies to better understand how community interactions might influence the effect of a contaminant. In doing so, it has become apparent that single species toxicity tests are likely over- or underestimating the true effects of contaminants on natural systems (Relyea and Mills 2001).

Numerous studies have demonstrated the effect of predator cues on pesticide lethality. For example, in a study by Relyea (2005), six species of frog tadpoles were exposed to varying concentrations of glyphosate and glyphosate combined with predator cues. Results suggested that at median concentrations, glyphosate lethality increased in the presence of predator stress. The effect of multiple stressors is not restricted to vertebrates. When *Daphnia* were exposed to both carbaryl and parasite stress, mortality was significantly higher than when they are exposed to either stressor individually (Coors and De Meester 2008). Additionally, a study by Hanazoto

and Dodson (1992) demonstrated that *D. pulex* exposed to carbaryl and predatory midge cues (*Chaoborus sp*) had delayed maturation relative to those exposed to only carbaryl. While studies on combined stressors demonstrate that the effect of predator cues of contaminant lethality is context dependent, research into the underlying cause is lacking. Relyea and Mills (2001) hypothesized that the combined stressors phenomenon could be due to an interaction between two stressors, wherein the general stress caused by the predator presence exacerbates the toxic effects of the contaminant. This general stress could be associated with the “flight or fight” response that results in a number of physiological effects including changes in hormone levels and energy metabolism (Selye 1950).

Although most research into the effects of predator cues on toxicity have used pesticides, sodium sulfate was used in this study partly because it is one waste product of coal mining and ore refining, which is relevant to the Appalachian region (Birge et al. 1989; Soucek and Kennedy 2005). However, major ion toxicity in the form of total dissolved solids (TDS) is a byproduct of other sources such as food processing, reverse osmosis, oil and gas refining, agricultural runoff, and groundwater remediation (Ingersoll et al. 1992; Mickley et al. 1996; Pillard et al. 1996; Tietge et al. 1997; Goodfellow et al. 2000). Major ions in TDS are ordinarily harmless at low concentrations relative to other contaminants such as pesticides, but can be detrimental to aquatic organisms at high concentrations (Chapman et al. 2000, Soucek and Kennedy 2005). For example, a study by Elphick et al. (2011) exposed numerous aquatic invertebrates and vertebrates to varying sulfate concentrations, and results indicated that sensitivity is dependent on species. Furthermore, Soucek and Kennedy (2005) demonstrated that even relatively low concentrations of sodium sulfate caused decreased fecundity in daphnids (*Ceriodaphnia dubia*). Field studies have provided evidence that the effluent from these coal facilities contains TDS

concentrations reaching levels that have been demonstrated to cause harm to aquatic organisms (Kennedy et al. 2005). Currently, major ions are regulated by some state environmental agencies but not federal agencies (USEPA 2016).

One major source of natural stress in aquatic ecosystems is predation. There are two broadly defined means of chemical communication by which prey may assess predator danger: (1) alarm cues and (2) kairomones (Chivers and Smith 1998). Kairomones are substances passively released as a product of metabolism by heterospecific predators that prey are able to identify, and can also involve dietary cues from predators consuming conspecifics (Ferrari et al. 2010). Conversely, alarm cues are chemical cues from damaged individuals by which conspecifics can detect potential predator presence (Chivers and Smith 1998). Alarm cues have been identified in several species of organisms across many taxa. Isopods, tadpoles, salamanders, and fish are all examples of groups with at least one species that responds to alarm cues with predator avoidance behavior (Rajchard 2006; Brown-Wilusz 2008; Spivey et al. 2015). In Ostariophysi fish, the alarm substance has been identified as a nitrogen oxide compound, now known as “Schreckstoff”, which is released when skin club cells are ruptured (Reed 1969). Although the substance has not been identified in amphipods, previous studies have demonstrated that chemical cues from injured conspecifics induce a predator avoidance response in *Gammarus* (Wisenden et al. 1999).

In this study, we tested the combined effect of predator cues on sodium sulfate sensitivity in amphipods. Response to predator cues was initially confirmed with behavioral assays. Toxicity tests were conducted with combined stressors in order to determine whether there was a differential lethal response to sodium sulfate when amphipods were also exposed to alarm cue

and kairomone. Additionally, oxygen consumption was measured in response to predator cues to determine whether there was a physiological stress response in short-term exposure trials.

Hypotheses:

1. Amphipods will decrease activity and increase time spent in refuge in response to both types of predator cues.
2. Sulfate sensitivity will increase when amphipods are exposed to either kairomone or alarm cue, relative to sulfate alone.
3. Oxygen consumption will increase when amphipods are exposed to both types of predator cues.

Methods

Animal Collection and Maintenance

Amphipods were collected by dip-netting from a spring-fed wetland in Elliston, Virginia (Montgomery County, 37.21417°, -80.23667°) and transported back to the laboratory at ETSU in coolers containing site water. Prior to collecting, a water quality multimeter (YSI, Yellow Springs, Ohio) was used to record temperature, dissolved oxygen, conductivity, salinity, and pH in the wetland. Water samples were also taken to the laboratory to determine total hardness and alkalinity (method 8226 and 8221, Hach 1998).

Upon arrival at the laboratory, amphipods were transferred to a communal housing tank that consisted of a modified living stream fitted with a Ranco chilling unit (Frigid Units Inc., Toledo, OH) The base panel of the stream was removed in order to reduce excessive flow, and a 19 L bucket was modified by replacing the bottom with a mesh cover to fit around the base of the chiller so that amphipods would not be drawn into the chiller circulating impeller. The stream was filled with 450 L of moderately hard water (MHW, 15° C, USEPA 2002) and supplied with two aerating Poret® foam biofilters (EMW, Limburg, Germany). Large rocks with a moss (not identified) were transported from the collecting site and placed in the stream to replicate the amphipods' natural environment. In addition to the moss, watercress (*Nasturtium officinale*) was also taken from the collecting site and used as food for the amphipods. A grow-light (Lights of America, Walnut, CA) was placed 10 cm above the chamber with a timer set on a 12:12 L:D cycle. Before any experiment, amphipods were acclimated to laboratory conditions for a minimum of six days. Experimental animals were only kept in the laboratory for a maximum of 12 days prior to the initiation of any trials.

Mosquitofish (*Gambusia affinis*) were collected from a stream in Phipps Bend Wildlife Area near Surgoinsville, Tennessee (Hawkins County, 36.47833°, -82.78667°), and used as predators in this study (ETSU Animal Care and Use Committee Protocol P150201). Organisms were collected with dip nets and transferred to plastic bags filled with site water in groups of ten. An aerator was placed in each bag, and bags were transported to the laboratory in a cooler. Mosquitofish were housed in 75-L aquaria (20 fish per chamber) filled with dechlorinated tap water and supplied with a biofilter. Fish were fed fish food flakes (Tetra, Melle, Germany) daily.

Stimulus Collection

Kairomone was collected from mosquitofish (*G. affinis*) by placing individually weighed individuals in aquaria containing moderately hard water at a concentration of 1 mg wet mass/100 ml MHW similar to Chapman et al. (2017). Each fish was then fed four amphipods and held for 24 hours. Water from the aquaria was then filtered with 5 µm filter cloth (Duda Energy, Decaunter, AL). Stimulus was stored frozen in 50-ml centrifuge tubes as 35-ml aliquots.

Alarm cue was collected from adult amphipods within 24 h of field collection by macerating 600 amphipods (~10.2 g tissue) with a mortar and pestle and adding this to 50 ml of moderately hard water. The resulting suspension was then filtered through 0.4 micron filter paper (Ahlstrom Filtration, PA), and diluted with 550 ml of moderately hard water. After the alarm cue was collected, it was placed into 10 ml centrifuge tubes in 6 ml aliquots and stored frozen for later use. During all experiments, amphipods were exposed to a concentration of 5% kairomone dilution and 1% alarm cue dilution. These exposure levels are within the range of those used in studies that examined the effect of such chemical cues on behavioral changes in other organisms (Wisenden et al. 2009; Spivey et al. 2015; Chapman et al. 2017).

Behavioral Assays

The methods used to analyze predator avoidance response were modified from Wisenden et al. (2009). A matched-pair experimental design was implemented in behavioral assays, wherein behavior was recorded during pre and post-stimulus periods. Experimental chambers consisted of 275-ml clear polystyrene cell culture flasks (Corning™, Corning, NY) that were modified by removing the top portion of the flask, resulting in a 7.5 x 3.5 x 9.0 cm chamber. A vertical line was drawn down the center of each container. Before each trial, sterilized black aquarium sand (1 cm deep) and 200 ml of MHW were added (Figure 1). Additionally, a piece of green tulle mesh (Falk Fabrics, Johnstown, NY) was placed on one side of the chamber as a refuge. Trials were performed at 15°C, same temperature used to maintain amphipods.

One amphipod was transferred to an experimental chamber at the beginning of each trial and given a five-min acclimation period. Following the acclimation period, 5 ml of MHW was added by dispensing from a 5-ml micropipette held approximately 1 cm above the surface of the water in the center of the chamber. A five-min pre-stimulus observation period began 30 s after this water was added to the chamber. At the end of the pre-stimulus period, one of three treatments solutions (alarm cue, kairomone, or control MHW) was introduced to the chamber in the same manner. The five-min post-stimulus observation period began 30 s after the stimulus was added. During the observation periods, behavior was quantified by time spent moving and time spent on the refuge side of chamber. Movement was defined as swimming, crawling on substrate, and crawling through the refuge. Amphipods were not fed during the behavior trials.



Figure 1. *Behavior Chambers.*

Lethality Trials with Combined Stressors

The second phase of this study examined the effect of predator cues on amphipod sensitivity to sodium sulfate (Na_2SO_4 , Fisher Chemical, Suwanee, GA) by conducting side by side 96-h toxicity bioassays of sodium sulfate alone and sodium sulfate plus either kairomone or alarm cue. Sulfate test concentrations were derived from initial range-finding tests and included a MHW control and 250, 500, 1,000, 2,000, and 4,000 mg SO_4/L . The concentration gradient was obtained by serial dilution of a 4.0 g/L sulfate (5.9146 g/L Na_2SO_4) stock. Each test concentration included four replicate test chambers (further described below). Adult amphipods were counted and placed in a 37 L aquarium with 20 L of MHW and an aerator 24 h prior to the beginning of lethality trials, during which they were not fed.

Following the 24-h period, amphipods were randomly selected in groups of five and placed into exposure chambers that consisted of 150-ml polypropylene beakers (Fisher

Scientific, Pittsburgh, PA) filled with 150 ml of MHW. To facilitate 100% water changes, a secondary beaker (hereon mesh beaker) of the same size was modified by removing the bottom of the beaker and attaching fine tulle mesh. In preparation for trials, 48 exposure chambers were initially filled with 150 ml of MHW and a secondary mesh beaker was placed in each chamber. Five adult amphipods were randomly placed in each chamber, and chambers were then randomly placed into either control or predator treatment groups. Four chambers were assigned to each concentration within the treatment groups. Bioassays consisted of paired treatment groups in which either control and alarm cue or control and kairomone were used simultaneously.

Following the initial setup, an additional set of 48 beakers was prepared with 150 ml of the appropriate sulfate concentrations (4 beakers of each concentration within each treatment group). Secondary mesh beakers containing amphipods were transferred from the preparation chambers to the test chambers with appropriate sulfate concentrations. Either control or predator cue was subsequently introduced to each chamber with a transfer pipette held 5 cm above the surface of the water. Amphipods were exposed to the treatment for 2 hours. Another set of chambers was prepared as previously mentioned, and amphipods were transferred into these chambers following the exposure period. The length of the exposure period was determined by preliminary data which indicated that amino acid breakdown in predator cue resulted in high ammonia levels after approximately 3 h (Appendix A).

Following the initial exposure, survival was recorded every 24 h. During each time check, dead amphipods were first checked for lack of response to a stimulus (jet of water from pipette followed by agitation with pipette) and then removed with a pipette. After counting and removing dead amphipods, treatment solution (either predator cue or MHW) was then added to the chambers for the each treatment group and left for the two-h exposure period. Mesh cups

were transferred to fresh water at corresponding sodium sulfate concentrations at the end of each exposure. Each assay was conducted in an incubator that was maintained at 15° C on a 12:12 L:D cycle.

At the end of each water change, one replicate exposure chamber was randomly selected from each concentration within each treatment group (control, alarm cue, or kairomone). For determination of dissolved oxygen, pH, and conductivity, measurements were taken in each of the replicates (YSI models 4010-2, 5000, 3100; Xylem Inc., Yellow Springs, OH). Additionally, one control (zero concentration) and one high concentration was used from each treatment group to measure water hardness and alkalinity (USEPA 2002). The high concentration used for hardness and alkalinity was not always a 4,000 mg SO₄/L group, as total mortality was often observed before 96 h. Burette titrations were performed to measure total hardness and alkalinity (method 8226 and 8221, Hach Company 1998) with standard solutions (Hach, Louisville, KY).

Influence of Predator Cues on Amphipod Metabolic Rate

The final goal of this study was to determine whether predator cues induced a change in metabolic rate of the amphipods. Oxygen consumption was used to quantify metabolic rate and was measured using a Firesting O₂ Fiber Optic Oxygen Meter (PyroScience, Aachen, Germany). Experimental chambers consisted of 20-ml vials (20ml Ox Vial, PyroScience) with self-healing septa that allowed for two 27 gauge syringe needles (Terumo, NJ) to be introduced for input and output flow. Trials were performed in a water bath kept in an incubator at 15° C. To maintain constant mixture within each experimental chamber, a multichannel magnetic stir plate (Thermo Scientific, MA) was placed below the water bath and micro stir bars were placed in each chamber (250 RPM). A 3x3 cm square of plastic mesh (Darice®, OH) was placed in each vial so that amphipods could avoid agitation from the magnetic stir bars.

At the beginning of each trial, five adult amphipods were placed in an experimental chamber and given a three-h acclimation period. The acclimation period was determined by preliminary trials that suggested oxygen consumption rates stabilized after amphipods had been in the chambers for at least two h. To maintain oxygen levels above 80% saturation, a peristaltic pump (Masterflex, Vernon Hills, Illinois) flushed the chambers (10 ml/min for 5 minutes) with oxygenated water every 55 min.

Following the three-h acclimation period, input tubes (pulling solution from a reservoir and pumping into chamber) were transferred to a randomly selected reservoir (2 L Nalgene® beaker) containing either control (MHW), kairomone, or alarm cue. All treatment solutions were prepared in the aforementioned manner, and diluted to obtain the respective concentration in a volume of 2 L. The subsequent flush initiated the two hour exposure period, during which dissolved oxygen data were collected every 10 s in 55-min increments (with 5-min flushes between increments) for a total of two h. During the final (12th) recording period, input tubes were transferred to a reservoir containing only MHW, and dissolved oxygen was measured for two additional h. Following each trial, all equipment was thoroughly rinsed with hot water, deionized water, and sterilized with a 70% ethanol solution to avoid bacterial growth.

Each group of amphipods was removed at the end of a trial and placed in a drying oven at 65° C for 24 h. Amphipods were subsequently placed in a desiccator jar for one hour. Dry mass was measured with a Sartorius CP225D balance (Sartorius AG, Göttingen, Germany) and recorded to the nearest 0.00001 g.

Statistical Analysis

Behavioral response to predator cues was analyzed using PROC GLM in SAS 9.4 (SAS Institute, Carey, NC). All comparisons were performed at $\alpha = 0.05$. The data used in the statistical analysis were obtained by subtracting the pre-stimulus period from the post-stimulus period for each observation. Normality was determined by Shapiro-Wilk tests on the residuals and examination of the normal Q-Q plot. Homogeneity of variances was verified with Levene tests (residuals) and also by regressing the residuals on expected values. A one-way Analysis of Variance (ANOVA) was used to test for treatment effect, with time spent active or time spent on refuge side as the response variable for the three treatment groups (alarm cue, kairomone, and control). A Tukey post-hoc analysis was used when an F -test indicated a significant difference ($\alpha = 0.05$) among treatment groups.

Survival data were analyzed by means of four statistical methods. First, a model comparison method, similar to that used in Seefeldt et al. (1995) and Oris and Bailer (1997), was used to determine whether slopes or intercepts differed between treatments. The model comparison method was performed using PROC LOGISTIC in SAS, and a model fit statistic was produced to determine whether data appropriately fitted the models. Three models were produced in a step-wise manner, and the error sum of squares (SSE) and degrees of freedom (DF) were obtained from each model to produce a full versus reduced F statistic (equation 1). First, a full model was produced that included both slope and intercept estimates for treatments separately. This model was used as the full model in all subsequent comparisons. Next, three reduced models were produced with null hypothesis that the excluded model terms were not different among treatments. For the first reduced model (hypothesis 1), both slope and intercept terms were removed. A significant F statistic comparing this model to the full model indicates

that either the slopes, intercepts, or both terms were significantly different among treatments. The second reduced model (hypothesis 2) excluded the intercept term to test the null hypothesis that intercepts did not differ. Finally, a third reduced model (hypothesis 3) was produced to test the null hypothesis of equal slopes among treatments. Refer to Appendix B for SAS code used in model comparison.

$$\frac{(SS_e^R - SS_e^F) / (DF_e^R - DF_e^F)}{(SS_e^F / DF_e^F)}$$

Equation 1. *F-test for model comparison.*

Superscript R indicates reduced model, F indicates full model.

Following the model comparison, LC₅₀ values were produced and compared with a ratio test. Confidence intervals were also produced with a multiple comparison method in R (3.2.5, package drc and multcomp). Third, a two-way ANOVA was used to determine significant differences among concentration of sulfate treatments. For the two-way ANOVA (PROC GLM, SAS), the zero and highest concentrations were excluded as there was no expected difference in these concentrations. Assumptions of normality and homogeneity of variances were checked with residuals using normality plots, residual versus fitted plots, Shapiro-Wilks, and Brown Forsythe test. Finally, an independent action (IA) analysis was used to visualize synergistic/antagonistic interactions among treatments. Expected values were produced according to Qin et al. (2011). IA plots were produced by plotting the expected values for each concentration and the mean response for the combined predator treatment group. We followed the interpretation described in Coors and De Meester (2008) such that if the estimated effect was

within the 95% CI of the observed value for the combined treatment, then the effect was additive; if the 95% CI fell below the estimated value, then the effect was considered synergistic.

Oxygen consumption data were analyzed by using the absolute value of the slopes (oxygen depletion over time) for the exposure and non-exposure periods of each treatment. Natural oxygen depletion caused by residual bacteria was accounted for by subtracting the slope of the empty vial from all others for each period. The dry mass from each group was used to calculate the mass corrected oxygen consumption data which were used in the final analysis.

Assumptions for normality and homogeneity of variances were checked using the aforementioned methods. Mass corrected oxygen consumption from the replicates for each treatment group in exposure and non-exposure periods were compared with a repeated measures ANOVA (PROC GLM, SAS). Differences among treatments at each period and within treatments at different periods were analyzed using a Bonferroni Post Hoc analysis ($\alpha = 0.05$).

Results

Behavioral Assays

Behavioral response to predator cues was quantified by time spent moving (activity) and time spent in refuge. Activity was significantly different among the three treatment groups ($F_{34,2,0.05} = 11.10$; $p = 0.0026$, Figure 2a). Activity in control and kairomone treatments were both significantly different from alarm cue ($p = 0.0001$, 0.0427 , respectively), but control and kairomone groups did not differ ($p = 0.195$). Amphipods exposed to alarm cue spent significantly less time moving in the post-stimulus period compared to other groups.

Time spent on the refuge side of the chamber was also significantly different among the treatment groups ($F_{34,2} = 7.12$; $p = 0.0026$, Figure 2b). For time spent in refuge, both alarm cue and kairomone were significantly different from control ($p = 0.0047$, 0.0332) but not from each other ($p = 0.784$). Amphipods exposed to either predator treatment spent significantly more time on the side of refuge than those exposed to the control.

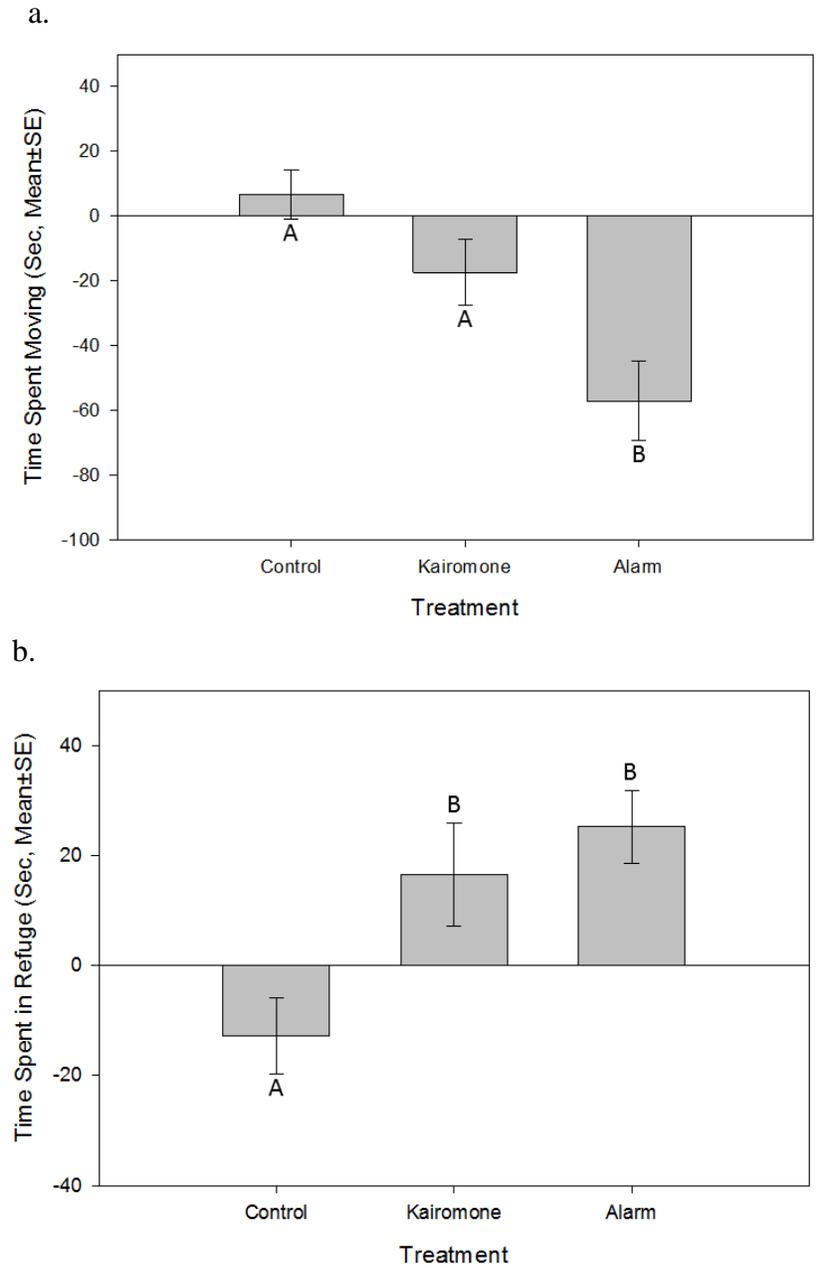


Figure 2. Behavioral Response to Predator Cues. Bars represent the difference in time spent moving (a) or time spent in refuge (b) between post and pre stimulus periods in control (n = 18), kairomone (n = 9), and alarm cue (n = 9) treatments. 2a, negative values indicate reduced activity in response to the treatment. 2b, positive values indicate increased time spent in refuge in response to the treatment. Difference in means (Tukey, $\alpha = 0.05$) are indicated by different letters.

Lethality Trials with Combined Stressors

The model comparison analysis did not indicate a difference in slopes in either the kairomone or alarm cue trials (Table 1), suggesting that LC_{50} estimates were reliable indicators of differences in sensitivity. In the kairomone trials, there was a significant difference in LC_{50} s between the kairomone and control groups (ratio test; Estimate = 0.355; $T = -2.838$; $p = 0.0051$). In this case, the LC_{50} for sulfate and kairomone combined was lower than that of sulfate only (Table 2). However, LC_{50} s in the alarm cue trials were not significantly different between alarm cue and control treatments (Estimate = 0.513; $T = -0.681$; $p = 0.498$).

Table 1. *Model Comparisons for Toxicity Tests.* A model comparison method was used to determine whether slopes and intercepts were different for lethality trials. For each model, the SSE and DF were used to compare with the full model. The three hypotheses are: (H1) slopes and/or intercepts differ, (H2) intercepts differ, (H3) slopes differ. In kairomone trials, the intercepts differ but slopes did not (a). Neither slopes nor intercepts differ in alarm cue trials (b) therefor values for H2 and H3 were excluded.

Treatment	Model	SSE	DF	F	P
Control vs. Kairomone	Full	3.5448	153	-	-
	H1	3.9023	156	5.14	0.002
	H2	3.6362	154	3.945	0.049
	H3	3.6288	154	3.626	0.059
Control vs. Alarm Cue	Full	2.349	113	-	-
	H1	2.372	116	0.369	0.776

Table 2. *LC50 Estimates.* Estimates for LC50s and 95% confidence intervals (mg/L) for each treatment were calculated using a multiple comparison package in R 3.2.5 (drc, multcomp).

Treatment	LC50 Estimate	95% CI
Control	479	232, 726
Kairomone	309	242, 376
Control	407	126, 688
Alarm Cue	226	138, 314

The overall F test for the two-way ANOVA model for the kairomone and control trials indicated a significant difference ($F_{120,7} = 30.28$; $p < 0.0001$, Figures 3a and 3b). Type I SS indicated an effect of treatment ($F_1 = 5.23$; $p = 0.024$), but the treatment by dose interaction was not significant ($F_3 = 2.23$, $p = 0.0878$). While the interaction effect was not significant, a Tukey post hoc analysis was used to examine differences between treatments at different concentrations. The only concentration at which treatments differed significantly was 0.5 g/L sulfate ($p = 0.0456$). The overall F test for alarm cue and control trials was significant ($F_{88,7} = 10.65$; $p < 0.0001$), but neither treatment ($F_1 = 0.4$; $p = 0.531$) nor treatment by dose interaction ($F_3 = 0.23$; $p = 0.878$) were significant.

Results from the IA model comparisons indicated a synergistic response in lethality when sulfate combined with kairomone at two concentrations (0.5 and 1.0 g/L sulfate, Figure 4a) and an additive response in sulfate combined with alarm cue at all levels of sulfate (Figure 4b).

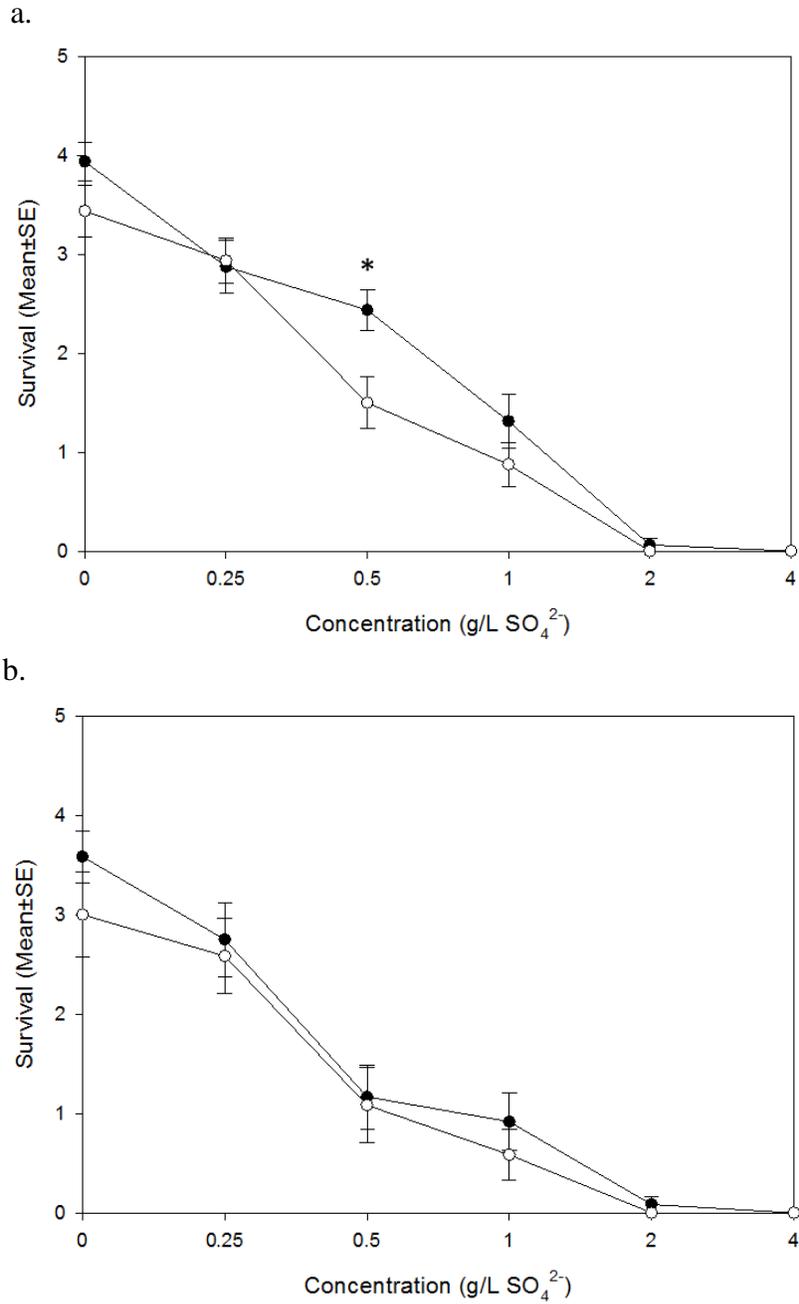


Figure 3. *Survival in 96 hour Lethality Trials with Combined Stressors.* Closed circles represent mean survival for control groups in both figures, open circles represent either kairomone (a) or alarm cue (b) exposures. Error bars represent standard error. * indicate significant difference in treatment at concentration ($\alpha=0.05$).

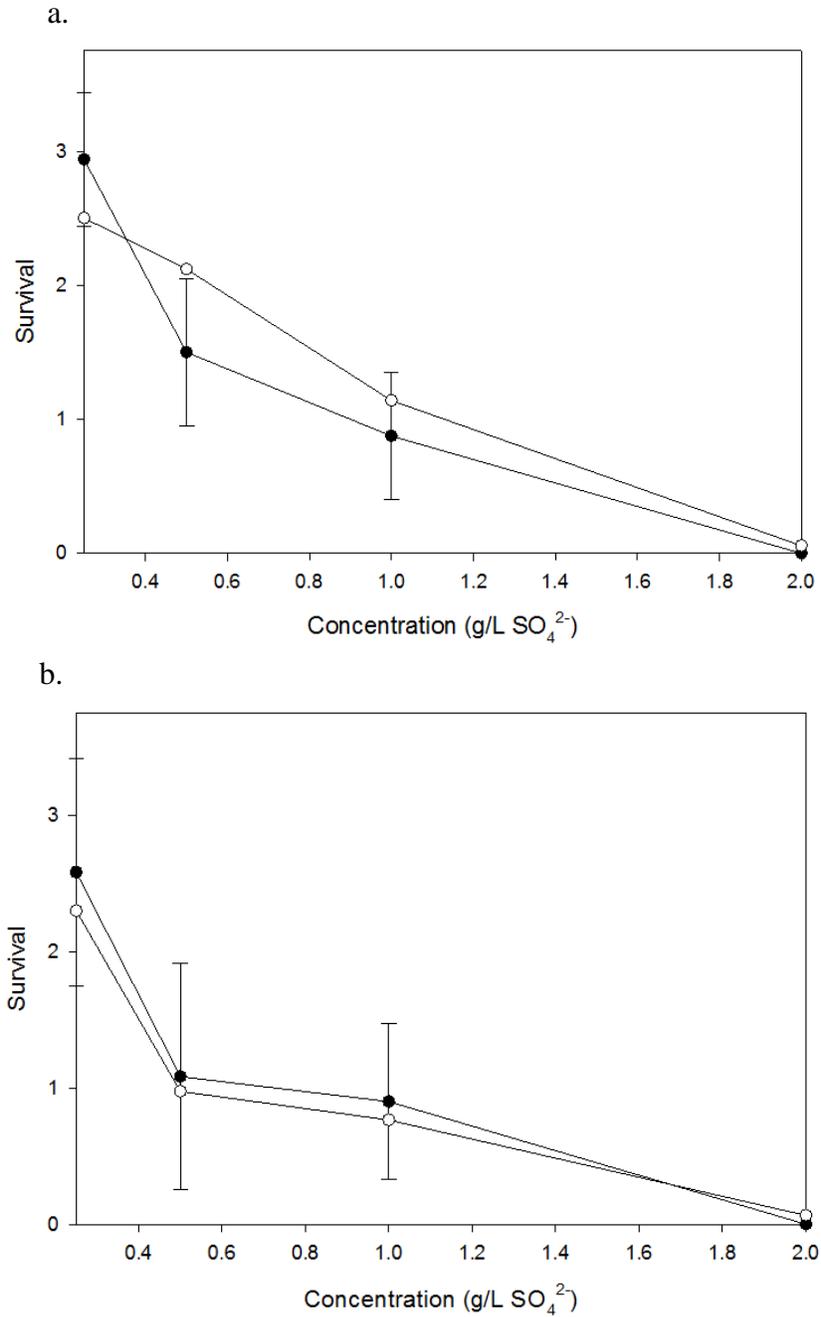


Figure 4. IA Models. Expected values were produced according to Coors and De Meester (2008). Closed circles represent mean survival for sulfate with predator treatment groups and error bars represent 95% confidence intervals. Empty circles represent expected values for the model. A synergistic response is observed at 0.5 and 1.0 g/L for kairomone trials (a), while the response to alarm cue was fully additive (b).

Metabolic Rate Trials

The overall F test for the Repeated Measures (RM) ANOVA indicated significance in the model ($F_{38,5} = 3.88$; $p = 0.0062$). The effect of treatment was significant ($F_2 = 6.16$; $p = 0.0048$), but neither period ($F_1 = 2.04$; $p = 0.162$) nor the treatment by period interaction ($F_2 = 2.51$; $p = 0.095$) were significant. Amphipods exhibited elevated oxygen consumption in response to kairomone as compared to control and alarm cue, and oxygen consumption within the kairomone treatment was significantly higher during the period of exposure relative to the non-exposure period (Figure 5).

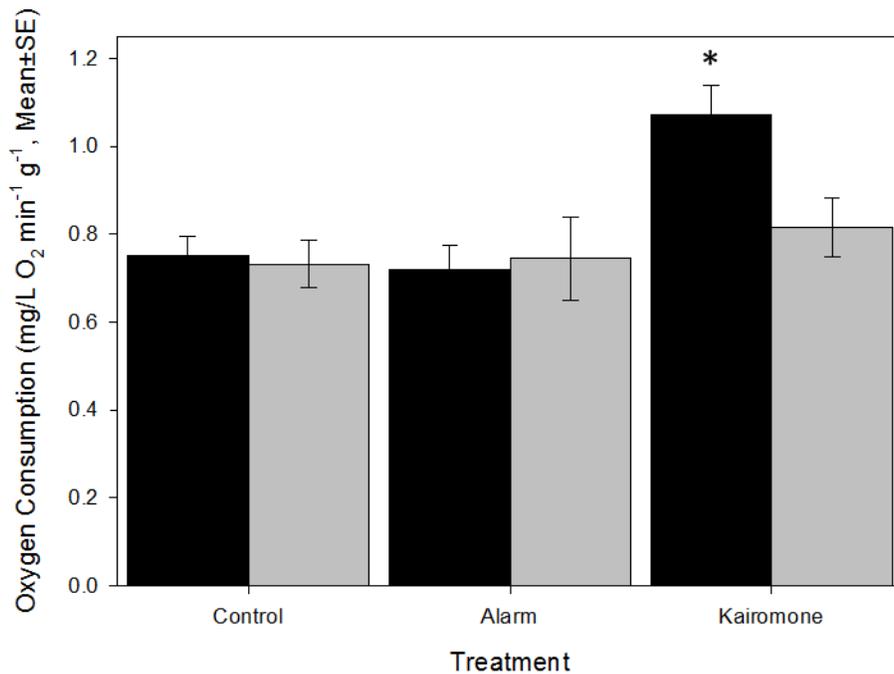


Figure 5. *Oxygen Consumption in Response to Predator Cues.* Bars represent mean mass corrected oxygen consumption during exposure (black) and non-exposure (gray) periods for each treatment. * indicates significance ($\alpha=0.05$) from post hoc analysis.

Discussion

Effect of Predator Cues on Behavior

The results of the initial behavior assays indicated that amphipods responded to both kairomones and alarm cues to varying degrees. The significant predator-avoidance response to conspecific alarm cue was not surprising, as numerous studies support this finding (Wudkevich et al. 1997; Wisenden et al. 1999; Wisenden et al. 2009; Sehr and Gall 2016). Alternatively, the basis for the response to *Gambusia* kairomone is less clear. While a number of studies indicate that amphipods respond to kairomones, the majority involve fish or other predator species from the same habitat as the amphipods (Mathis and Hoback 1997; Wooster 1998; Åbjörnsson et al. 2000). The amphipod population used in this study was unique in that they exist in a habitat that is most likely void of fish predators.

A study by Åbjörnsson et al. (2004) analyzed the behavioral response of amphipods (*Gammarus pulex*) from ponds both with and without fish predators. The results indicated that while amphipods from ponds with fish demonstrated predator avoidance behavior when exposed to crucian carp (*Carassius carassii*), those from fishless ponds did not. However, the fish used in their study were not fed amphipods and ours were. The behavioral response to cues from *Gambusia* was different from the alarm cue response in that amphipods increased time spent in refuge, but did not significantly decrease activity relative to the control group. One explanation for this finding could be that dietary cues paired with fish kairomone induced a predator-avoidance response, albeit slightly weaker than the response to alarm cue. A study by Åbjörnsson et al. (2000) demonstrated that diet did not have an effect on behavioral response when *G. pulex* were exposed to fish fed conspecifics, heterospecifics, or starved. However, the amphipods used in their study were from a habitat with fish predators. Schoeppner and Relyea

(2009) found that the presence of alarm cue or dietary cue enhanced the response to predator kairomones in Leopard frog tadpoles (*Rana pipiens*). It is possible that although predator diet does not influence the response of amphipods regularly exposed to predators, dietary cues could induce a response to novel predator cues in other populations that are not exposed to predators.

Another reason for the weaker response to kairomone observed in this study could be that the response is due to residual alarm cue that was present in fish water as a result of mechanical damage during consumption. However, a study by Wisenden et al. (2009) demonstrated that alarm cue in *Gammarus lacustris* induced a significant response within 3 h of exposure, but amphipods did not respond to cue that was 6 h old. Additionally, preliminary results from this study indicated a significant increase of ammonia in water containing alarm cue after 3-6 h. This supports the hypothesis proposed by Wisenden et al. (2009) that the active alarm cue likely contains proteins, which degrade over time. The *Gambusia* in our study were fed only five amphipods 24 h prior to harvesting the treatment water, and in each case the fish had consumed all amphipods after 12 h. Due to the fast protein degradation, it is unlikely that residual active alarm cue remained when kairomone was harvested.

Effect of Predator Cues on Oxygen Consumption

Amphipods exhibited increased oxygen consumption in response to predator kairomones but not to alarm cue. These results contradict the findings from behavior assays in which alarm cue induced a stronger predator avoidance response. Although there is currently no literature on amphipod metabolic rate in response to alarm cue, some work has been done on the response of amphipod oxygen consumption to kairomone. For example, Glazier et al. (2011) demonstrated that the resting metabolic rate for *Gammarus minus* from streams with fish predators was lower than those without fish predators. While this does not support the increased oxygen consumption

observed in response to kairomone in this study, it is possible that the metabolic rate response to predator stress is different between long-term and short-term exposure regimes. In many organisms, short-term exposure to predator cues induces a “fight or flight” response and increased metabolic rates, while long-term exposure to predators results in suppressed metabolic rates. This has been observed in fish (Holopainen et al. 1997; Hawkins et al. 2004) and amphibian larvae (Steiner and Van Buskirk 2009). It has been hypothesized that suppressed metabolic rate in response to long-term predator exposure could be an adaptation to compensate for reduced foraging and other negative effects of predator avoidance behaviors (Werner and Anholt 1993; McPeck 2004), though Steiner and Van Buskirk (2009) did not find evidence to support this in tadpoles (*Rana temporaria*)

Although alarm cue did not influence oxygen consumption as would be expected from behavior trials, it is possible that the behavioral response to different types of predator cues is not consistently linked to specific physiological pathways. A study by Schoeppner and Relyea (2009) demonstrated a complicated relationship between behavioral and morphological response to alarm cues, predator cues, and their combinations in leopard frog tadpoles (*Rana pipiens*). It is also possible that the lack of response in activity alone to kairomone does not indicate a weaker predator-avoidance response, but rather a different form of avoidance behavior. For example, in the previously mentioned study by Schoeppner and Relyea, tadpole activity and time spent in refuge were quantified in response to different predator cues. Tadpoles increased time in refuge only in response to alarm cue, but decreased activity only in response to dietary cues. Interestingly, only dietary cues induced an anti-predator morphological response. In conclusion, the authors suggested that the differences in behavioral and morphological response to different types of cues could reflect the decision between fast and reversible behavioral response versus

slow morphological response. This suggestion also relates to previous findings that demonstrated stronger behavioral response to paired alarm cue and kairomone than to alarm cue alone. Further research is necessary to better understand the interactions between behavioral and physiological response to different types of predator cues.

Effect of Predator Cues on Sulfate Lethality

The 96 h LC₅₀ estimate for sulfate alone (~480 mg/L) is comparable with previous sulfate toxicity studies using amphipods from the genus *Hyalella* (Soucek and Kennedy 2005). While LC₅₀ estimates for kairomone and alarm cue exposures were not significantly different from the controls, further investigation of survival curves indicated a significant effect of kairomone at median concentrations of sulfate. Results from the two-way ANOVA suggested that survival in the kairomone treatment at 500 mg/L sulfate was significantly lower than the control. Additionally, the IA analysis indicated a synergistic interaction at both 500 and 1,000 mg/L sulfate concentrations. This method of analyzing dose response curves in addition to point estimates was suggested by Qin et al. (2011), who stated that sensitivity can differ at some concentrations but not others and that this could explain variation in results across different studies. This argument applies to our study, and could have implications for risk assessment when considering the effects of combined stressors. Point estimates alone suggest that predator cues had no effect on sulfate sensitivity. However, analysis of dose response curves clearly show that predator kairomones increase sulfate sensitivity at concentrations around 400-1,000 mg/L. A study by Sidle et al. (2000) found high frequencies of riverine ponds with sulfate concentrations at 400-500 and 800-900 mg/L in Indiana and Illinois. Given that these values are within the range of synergistic interactions shown in our study, this is a significant example in which single

species toxicity tests would likely underestimate the true impact of sulfate at environmentally relevant concentrations.

Studies with combined stressors that evaluate contaminant lethality with predator stress of both alarm cues and kairomones are lacking. However, the study by Robison et al. analyzed the effects of alarm cue, kairomone, and both combined on either sodium chloride or cadmium chloride sensitivity in *Pimephales promelas*. They found that alarm cue produced the most significant effect on sodium sensitivity, followed by alarm cue paired with predator kairomone. Predator kairomone alone did not influence sensitivity. The fact that alarm cue paired with kairomone did induce a response is not surprising. Since alarm cues from injured conspecifics could be strongly associated with a predation event, an enhanced response to both alarm cue and kairomone combined could allow prey to avoid reacting to kairomone even when there is no risk of predation (Chivers et al. 2013; Mitchell et al. 2015), Schoeppner and Relyea 2009). Thus, it is not surprising that response to a predator kairomone could be developed through associative learning with alarm cue (Mitchell and McCormick 2013; Atherton and McCormick 2015). Relyea (2004) demonstrated that predator stress does not necessarily influence contaminant sensitivity of different prey species from the same habitat. In the study comparing the effects of predator kairomones on six species of tadpoles, point estimates for combined stressors were highly variable. Additionally, there was only a pesticide-predator interaction in one of the six species. Results from these studies suggest that the interaction between predator stress and contaminant lethality is complex and highly dependent on the model organism, predator species, and type of contaminant.

The experimental design for combined stressor bioassays in this study is unique in that amphipods were only exposed to predator cues for two hours each day. All previous studies have

involved constant predator cue exposure. Protein degradation and ammonia buildup from the alarm cue prevented 24-hour exposure in this study. Thus, the exposure period for kairomone was also reduced to two hours to allow for comparison among the treatment groups. To our knowledge, this is the first study to demonstrate that chemical cues used in predator-prey interactions influence water quality. However, previous studies have demonstrated that alarm cue from amphipods induces a weaker predator avoidance response after 3 h, and is ineffective at 6 h (Wisenden et al. 2009; Ferrari et al. 2010). It is unlikely that alarm cue would remain static and not diffuse for long periods of time in a natural environment, but this finding could be a cautionary note for future research in the field. If the goal of a study is to assess the effect of predator cues on sensitivity to a contaminant, significant variation in water quality between treatments could be a confounding variable that introduces a third stressor. The results could indicate a significant effect of predator cues when none actually exists.

Conclusion

Our study demonstrated that metabolic rates, but not behavior, in response to predator cues could help explain the results of the combined stressor bioassay with sulfate. Amphipods increased oxygen consumption in response to *Gambusia affinis* kairomone and also increased sensitivity to sulfate when kairomone was present. Ion regulation is an energetically costly event that is linked to oxygen consumption (Morgan and Iwama 1999; Tseng and Hwang 2008). Increased metabolic rate in response to predator kairomone could deplete energy stores that are normally allocated to maintenance and ion regulation at a faster rate, leading to higher mortality in response to the combined stress at mid-range concentrations. In contrast, alarm cue did not influence metabolic rate nor sulfate sensitivity. However, both types of predator cues induced a predator-avoidance response. To our knowledge, this is the first study of combined stressors to

incorporate preliminary behavioral trials. Although behavioral response to different cues did not explain differences in sulfate sensitivity in our study, it is possible that the type of behavioral response is also important. For example, reduced activity in response to alarm cue might cause a decrease in metabolic rate enough to offset the effects of the fight or flight response observed with kairomones. Future studies incorporating behavioral assays that quantify more response variables may be helpful in better understanding the interactions between behavioral and physiological responses to different predator cues.

Research demonstrating interactions between predator stress and contaminant lethality challenges the utility of single-species toxicity tests currently used to regulate anthropogenic stressors in natural environments. Although the results of our study and similar research is variable, incorporating metabolic rates into future studies could help explain why synergistic interactions are observed in some contexts but not others. Additionally, the contradictory results of our behavior assays raise new questions to be addressed in this area of research.

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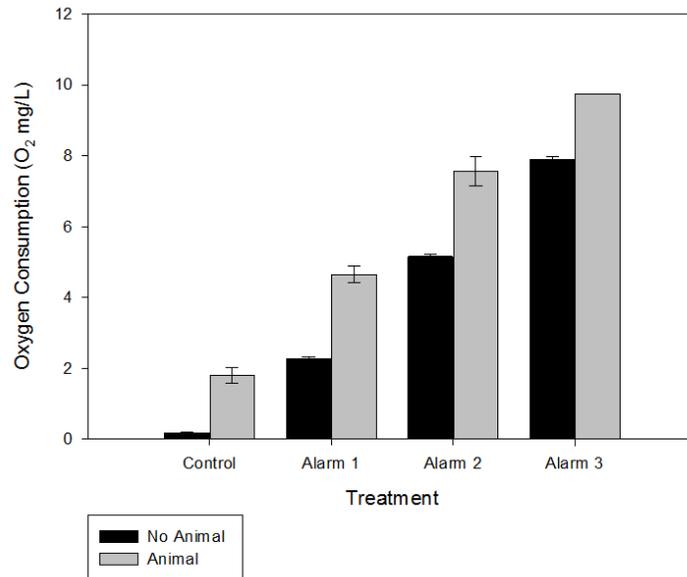
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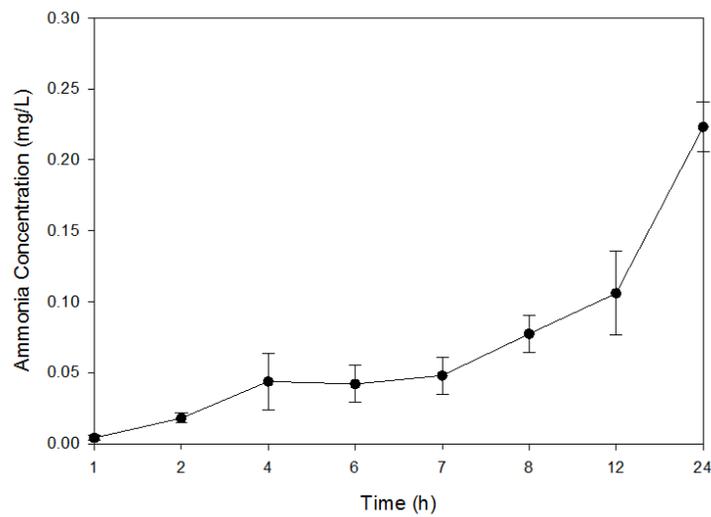
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APPENDIX A: EFFECTS OF ALARM CUE ON WATER QUALITY

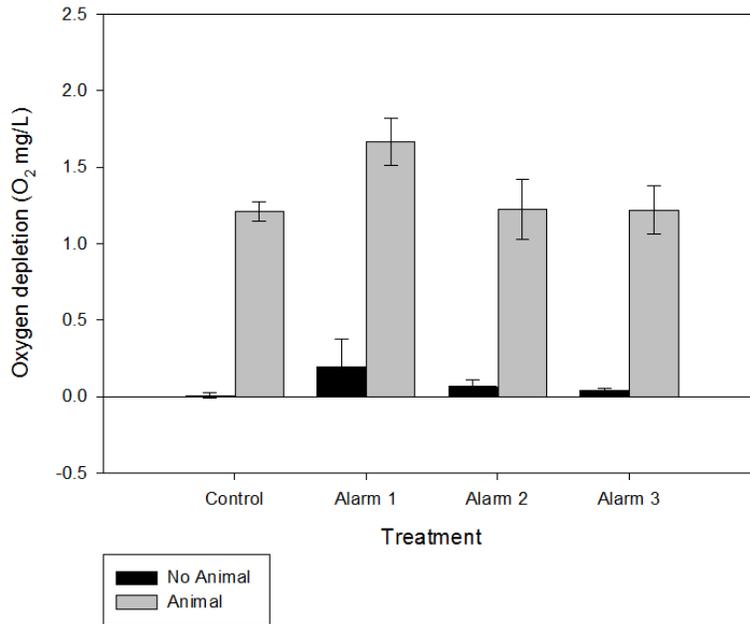
Prior to conducting oxygen consumption trials with the FireSting O₂ oxygen meter, BOD bottles were used to compare oxygen consumption in amphipods exposed to varying concentrations of alarm cue (1 individual/100 ml, 3/100ml, 5/100ml). Alarm cue was prepared as described in the stimulus collection section of the methods. In order to account for any oxygen depletion caused by residual bacteria in the alarm cue, empty BOD bottles with either control or alarm cue concentrations were run parallel to amphipod replicates. At the beginning of each trial, BOD bottles were filled with MHW containing each concentration of alarm cue and initial dissolved oxygen was measured with a BOD probe (YSI model PRO20). Five amphipods were introduced to half of the replicates, and the bottles were then sealed and placed in an incubator at 15°C. Dissolved oxygen was recorded after 24 hours. Results from these trials demonstrated that while oxygen consumption appeared to increase at each level of exposure, it was actually proportional to the oxygen depletion occurring in the bottles with no amphipods (below).



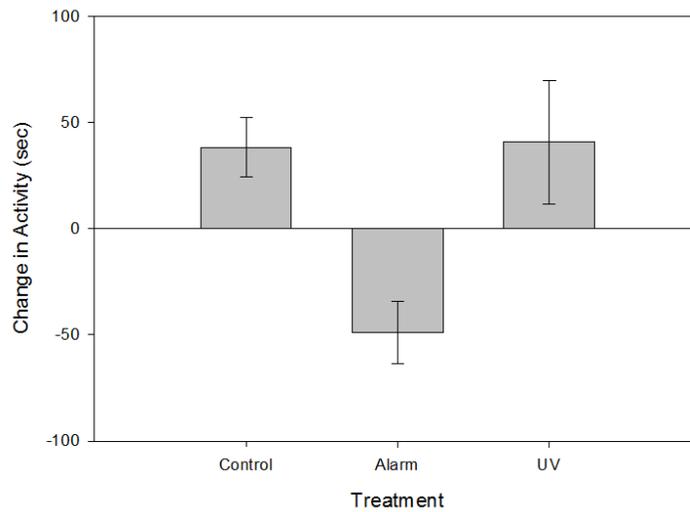
One of our hypothesis for the depleted oxygen was that bacteria were degrading the proteins in the cue. To test this, we measured ammonia content over a 24 hour period with the median concentration of alarm cue. Previously described methods were used to prepare alarm cue, and 100 ml of was added to a 200 ml beaker. Samples were measured at 2, 4, 6, 7, 8, 12, and 24 h with a MultiLab IDS probe (YSI model 4010-2). Ammonia began to increase between 1 and 2 h, and continued to increase over 24 h (below).

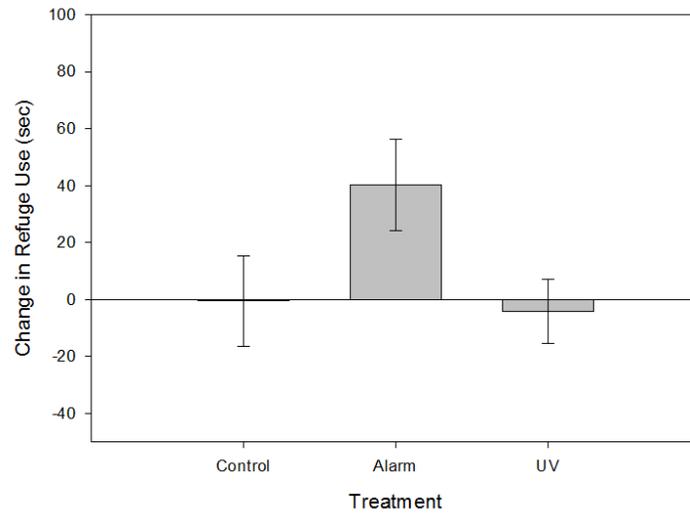


To address the problem of oxygen depletion and high ammonia content caused by alarm cue, we attempted to filter the cue with filter cloth (described in methods) and applied a UV light (AA Aquarium, Kwun Tong, Kowloon) for one hour prior to trials. After preparing the cue, trials were conducted with BOD bottles as described previously. Results indicated that the filtering method alleviated the ammonia problem, but there did not appear to be a response at any concentration of alarm cue (below).



We tested whether the cue was still active after the filtering process by performing behavioral trials as described in the methods of this paper. Amphipods were exposed to control, alarm cue, and UV filtered alarm cue. While the alarm cue still induced a behavioral response, amphipods neither reduced activity or increased time spent in refuge when exposed to UV treated cue (below: top, activity; bottom, refuge use).





APPENDIX B: SAS CODE FOR MODEL COMPARISON ANALYSIS

```
data dkair;
input block  treat  dose  surv  propsurv  tsurv;
ldose=log(dose);
cards;

proc glm data=dkair;
class dose treat;
model propsurv=dose treat dose(treat)/ ss1 ss2 solution;
run;

proc nlin data=dkair;
parameters
d=.7
c=0.0
i50=.6
b=2.5
;

if treat='1'
then predict=c+(d-c)/(1+exp(b*(ldose-log(i50))));
else if treat='0'
then predict=c+(d-c)/(1+exp(b*(ldose-log(i50))));
model propsurv=predict;
run;

/*full model*/
proc nlin data=dkair;
parameters
d_1=.57
d_0=.95
c=0.0
i50_1=.93
i50_0=.33
b_1=3.4
b_0=1.6
;

if treat='1'
then predict=c+(d_1-c)/(1+exp(b_1*(ldose-log(i50_1))));
else if treat='0'
then predict=c+(d_0-c)/(1+exp(b_0*(ldose-log(i50_0))));
```

```

model propsurv=predict;
run;

/*equal upper limit*/
proc nlin data=dbg;
parameters
d=.8
c=0.0
i50_1=.93
i50_0=.33
b_1=3.4
b_0=1.6
;

if treatment='1'
then predict=c+(d-c)/(1+exp(b_1*(ldose-log(i50_1))));
else if treatment='0'
then predict=c+(d-c)/(1+exp(b_0*(ldose-log(i50_0))));
model propsurv=predict;
run;

/*parallel slopes*/
proc nlin data=dkair;
parameters
d=.9
c=0.0
i50_1=.93
i50_0=.33
b=2.5
;

if treat='1'
then predict=c+(d-c)/(1+exp(b*(ldose-log(i50_1))));
else if treat='0'
then predict=c+(d-c)/(1+exp(b*(ldose-log(i50_0))));
model propsurv=predict;
run;

```

VITA

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collected in Virginia. *Virginia Journal of Science*, in press,
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other chemical stressors in fathead minnows (*Pimephales
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Predation risk assessment by eastern newts (*Notophthalmus
viridescens*) in response to common snapping turtles and
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Trevor L. Chapman, Kari L. Vollmer, Brian G. Gall. March 2013.
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