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Impacts of ammonia and temperature on freshwater snail behavior and physiology.

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IMPACTS OF AMMONIA AND TEMPERATURE ON
FRESHWATER SNAIL BEHAVIOR AND PHYSIOLOGY

By

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B.S., Belmont University, 2012
M.S., University of Louisville, 2016

A Dissertation
Submitted to the Faculty of the
College of Arts and Sciences of the University of Louisville
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in Biology

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A Dissertation Approved on

November 5, 2018

by the following Dissertation Committee:

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Dr. Perri Eason

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Dr. Steve Yanoviak

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DEDICATION

I dedicate this dissertation to my husband

Michael DeWhatley

whose support and belief in me has made this work possible

and to my parents

Del and Jackie DeVries

who taught me that things worth having are worth working for.

ACKNOWLEDGEMENTS

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ABSTRACT

IMPACTS OF AMMONIA AND TEMPERATURE ON
FRESHWATER SNAIL BEHAVIOR AND PHYSIOLOGY

Megan Christine DeWhatley

November 5, 2018

Gastropods are one of the most imperiled groups of animals in North America, yet there are major gaps in the literature pertaining to pollutants and climate change, and especially sublethal impacts. This dissertation assesses the effects of climate warming and unionized ammonia (NH_3), one of the most abundant water pollutants, on the behavior and physiology of two caenogastropod snails: fine-ridged elimia (*Elimia semicarinata*) and Shawnee rocksnails (*Lithasia obovata*) (Gastropoda: Neotaenioglossa: Pleuroceridae). Righting behavior, or the movement used by snails to turn themselves right-side-up, was used as the main endpoint; delays in this behavior compromise fitness via lost feeding time and increased predation risk. NH_3 experiments involved acute (24 hr) exposure to a range of concentrations, with righting time tested before and after exposure. NH_3 significantly affected the change in righting time for fine-ridged elimia, with snails exposed to higher doses righting more slowly after exposure than before. Shawnee rocksnails did not experience this effect, but significantly more snails failed to right within the time limit (60 min) after exposure to 12.61 mg $\text{NH}_3\text{-N/L}$. The concentrations that affected the behavior of these species are magnitudes greater than any reported previously for freshwater gastropods. Oxygen consumption was also tested after

acute exposure to NH_3 to determine if righting behavior is affected via changes to respiration, but no effects were found, suggesting that this behavior is altered by a different mechanism, at least during short-term exposure. Temperature experiments involved chronic (10-day) exposure, with righting time tested before and after. Both species failed to right at greater proportions than controls (20°C) at temperatures below their streams' current summer highs; this effect could leave snails stranded on dry shores as water level fluctuations increase with climate change. Survival was drastically reduced at 35°C for fine-ridged elimia and 30°C for Shawnee rocksnails, 5°C less than the lower end of a common generalization of gill-breathing snail thermal tolerance. This research illustrates the importance of studying a wide range of species to determine tolerances for freshwater gastropods; conservation efforts cannot be properly informed without an understanding of the variation in sensitivities.

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INTRODUCTION

In the United States and Canada, 74% of gastropods are listed as vulnerable, threatened, endangered, or extinct (Johnson et al. 2013); a value that far exceeds the imperilment of fish (39%, Jelks et al. 2008) and crayfish (48%, Taylor et al. 2007). Conservation challenges range from declining freshwater quantity and quality to competition with invasive species, though a continued lack of efficient propagation and reintroduction techniques contributes as well (Lysne et al. 2008). The potential to lose almost 75% of the current gastropod diversity presents concerns for freshwater ecosystems. Snails provide several important ecological functions, including limiting the biomass of a complex assemblage of algae and bacteria, called periphyton, that covers submerged surfaces (Hill 1992; Brown et al. 2008). Additionally, snails aid the decomposition process in streams: leaf litter breakdown rates are positively correlated with gastropod species richness as well as biomass (Chadwick et al. 2006). Like other taxa, mollusk communities are more resistant to invasion when they are more diverse (Kennedy et al. 2002). For all of these reasons, the Freshwater Mollusk Conservation Society has called for more research on gastropod sensitivities, and particularly for comparative studies such as the ones contained in this dissertation (FMCS 2016).

Historically, toxicology research has been focused on lethal impacts and lethal concentrations or temperatures for environmental stressors and pollutants. However,

scientists have known for decades that fitness is compromised by impairments to a wide range of behaviors and physiological factors (Cohn and MacPhail 1996; Pyle and Ford 2017). Furthermore, behavioral endpoints are often between 10 and 100 times more sensitive than survival (Gerhardt 2007). Regardless of whether a behavior can function as an indicator of poor environmental quality, as many do (Hellou 2011), it is critical for protecting the diversity of freshwater gastropods that the sublethal effects of pollutants and climate warming be thoroughly studied so that conservation efforts can be as informed as possible (FMCS 2016).

In an effort to begin to fill the gaps in freshwater gastropod research, two important environmental stressors were chosen for analysis: unionized ammonia (NH_3) and climate warming. At present, both stream water temperatures and concentrations of NH_3 are rising due to human impacts (Durance and Ormerod 2009; Isaak et al. 2012; USEPA 2013). It is the aim of this dissertation to determine how levels of these factors that may be experienced by freshwater gastropods now or in the near future will affect the behavior and physiology of two freshwater gill-breathing snails, fine-ridged elimia (*Elimia semicarinata*, Say 1829) and Shawnee rocksnails (*Lithasia obovata*, Say 1829).

CHAPTER I

EFFECTS OF UNIONIZED AMMONIA ON RIGHTING BEHAVIOR AND RESPIRATION IN TWO PLEURO CERID FRESHWATER SNAILS

Introduction

Ammonia is a natural component of the nitrogen cycle, but both point and nonpoint sources have increased ammonia concentrations above natural levels in recent years (USEPA 2013). Industrially, ammonia is used for fertilizers, household chemicals, printing processes, metals extraction in mining, crude oil processing (USEPA 2004), and the production of pharmaceuticals and dyes (Karolyi 1968; Appl 1999). Agricultural runoff is a major source of ammonia pollution to freshwaters, especially in the form of synthetic fertilizers (Boyer et al. 2002; Howarth et al. 2012) and animal waste from livestock operations (USEPA 2004). The anthropogenic sources of ammonia listed above are in addition to natural sources such as animal waste from wildlife, nitrogen fixation (USEPA 2013), and the decomposition of organic matter (Russo 1985).

In aquatic ecosystems, ammonia exists in two forms: ionized (NH_4^+) and unionized (NH_3) (Abel 1996). In general, ammonia is more toxic than other components of the nitrogen cycle, such as nitrite and nitrate (Romano and Zeng 2013), but NH_3 is even more toxic than the ionized form (Abel 1996). Furthermore, increases in water temperature and pH cause the concentration of NH_3 to increase and the concentration of

NH_4^+ to decrease, making warmer, more basic waters more toxic to aquatic organisms when ammonia is present (Abel 1996). Depending on the taxa, ammonia can cause death to aquatic animals via collapse of the gill lamellae and other gill damage (Romano and Zeng 2013), a decrease in gill ventilation (Lang et al. 1987), degeneration of kidneys, or repression of the immune system, among other effects (Russo 1985).

Toxicants like ammonia often affect physiological processes and behaviors at concentrations far below lethal levels (Gerhardt 2007) and these effects can still have strong implications for fitness if behaviors like predator avoidance, food acquisition, or reproduction are affected (Alonso and Camargo 2013). The list of behaviors known to be affected by NH_3 include locomotor activity (in amphipods, Normant-Saremba et al. 2015), microhabitat choice (in amphipods, Gergs et al. 2013), movement behavior (in planaria, Alonso and Camargo 2015; in freshwater snails, Alonso and Camargo 2009), and feeding activity (in amphipods, Alonso and Camargo 2004; in shrimp, Frías-Espericueta et al. 2000). Movement behaviors in particular have become an area of focus for behavioral toxicology because there is a tight connection between an organism's ability to move (whether at all or at a regular pace) and its ability to perform behaviors required for foraging, mating, and avoiding predators (Alonso and Camargo 2013). A variety of toxicants impair movement in aquatic invertebrates (e.g. heavy metals, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons, Salánki et al. 2003) and research is beginning to show similar effects of NH_3 (see above). However, few studies have assessed the impacts of NH_3 on the behavior of freshwater gill-breathing snails.

This lack of research is surprising for two reasons. First, freshwater gill-breathing snails have been shown to be relatively sensitive to ammonia in toxicity tests (USEPA 2013), and second, gastropods are considered one of the most imperiled taxa in North America. Currently, 74% of gastropods in the United States and Canada are listed as vulnerable, threatened, endangered, or extinct (Johnson et al. 2013), as compared with 39% for fish (Jelks et al. 2008) and 48% for crayfishes (Taylor et al. 2007). Aquatic snails are important to aquatic ecosystems because they provide the vital ecosystem function of decomposition; snail species richness and biomass are positively correlated with leaf litter breakdown rates in streams (Chadwick et al. 2006). Snail grazing also regulates and limits the biomass of periphyton, a complex mixture of algae and bacteria that attaches to submerged surfaces (Hill 1992; Brown et al. 2008). The most recent strategy document by the Freshwater Mollusk Conservation Society makes an urgent call for data on sublethal effects of stressors for mollusks (FMCS 2016).

While all movement behaviors have the potential to impact snail fitness, a behavior called righting time is particularly important. This behavior represents the amount of time it takes an organism to “right” itself after it has been flipped upside-down with its dorsal surface facing upward; it has been studied in an array of aquatic animals (e.g. turtles, Domokos and Várkonyi 2008; sea stars, Lawrence and Cowell 1996; crayfish, Newland 1989; sea urchins, Challener and McClintock 2013) including freshwater and marine snails (Weldon and Hoffman 1979; Brown et al. 1998; Fei et al. 2007). Increased righting time is used as an indicator of stress in various gastropods (e.g. Weldon and Hoffman 1979; Fong et al. 2017) because snails that are flipped upside down, with the aperture, or shell opening, exposed, may be more vulnerable to predators

(Orr et al. 2007, Lemmnitz et al. 1989) and cannot carry out normal activities like mating or foraging. Several factors have been found to impact righting behavior in gastropods. For instance, both pH and temperature affect righting time, in an interactive way, in the marine snail, *Margarella antarctica* (Schram et al. 2014). Elevated temperatures also impair righting ability in the freshwater snails fine-ridged elimia (*Elimia semicarinata*) and Shawnee rocksnail (*Lithasia obovata*) (DeWhatley and Alexander 2018).

Although righting behavior is not a new method for assessing the impacts of stressors on aquatic gastropods, the behavior has not widely been used with toxicants. Another sublethal endpoint indicative of organismal stress and in need of further testing with regards to toxicants relates not to behavior but to physiology. Oxygen consumption is one of the most basic measures of animal health and proper physiological function. Oxygen is the final electron acceptor in the electron transport chain of aerobic respiration; as more energy is needed, oxygen demand increases as well. Thus, increases in an organism's oxygen consumption relate to increases in energy demand, which—other factors being equal—may indicate that the organism is stressed and requires additional energy to maintain physiological processes. There is a positive correlation between water temperature and oxygen consumption for many species, including freshwater mussels (Ganser et al. 2015) and freshwater snails (Hawkins and Ultsch 1979; Alexander and Wagoner 2016).

Similarly, oxygen consumption in the zebra mussel (*Dreissena polymorpha*) increases with temperature, but only up to about 30°C, after which the rate declines as the temperature continues to increase and the animal succumbs to heat stress (Alexander and McMahon 2004). Thus, declines in oxygen consumption can indicate impaired

physiological function. Such declines are seen when various taxa are exposed to lethal concentrations of toxicants, in the days prior to the organism's death (Bharathi and Rao 1989; Sivaramakrishna et al. 1991). Acute exposure to mercury causes a steep decline in oxygen consumption for the freshwater snail *Pila globosa* and the mussel *Lamellidens marginalis* (Sivaramakrishna et al. 1991). Other toxicants have a similar effect, including copper exposure for the marine prosobranch snail *Babylonia lutosa* (Cheung and Wong 1998) and aluminum exposure for *Daphnia magna* and two perlid stoneflies, *Perlestra lagoi* and *Acroneuria abnormis* (Soucek 2005).

This study used two laboratory experiments to test whether acute exposure (24 hours) to NH₃ affects righting behavior and oxygen consumption in two freshwater caenogastropod snails: fine-ridged elimia (*Elimia semicarinata*, Say 1829) and Shawnee rocksnails (*Lithasia obovata*, Say 1829; both Gastropoda: Neotaenioglossa: Pleuroceridae). Acute exposure was chosen because, in this instance, it is more environmentally relevant than chronic exposure; NH₃ concentrations in small streams and rivers are generally low on average, but spike to higher levels during pulses attributed to factors like rain (Yahdjian and Sala 2010), animal die-offs (Cherry et al. 2005), or rewetting of sediments (Baldwin et al. 2005). For the first experiment, I predicted that righting time would increase at higher concentrations of NH₃ as the snails' ability to move became impaired. Given that snails are naturally exposed to low concentrations of NH₃, I also predicted that the effects on oxygen consumption would more closely mirror those of temperature than heavy metals such as mercury and aluminum. Thus, for the second experiment, I expected that acute exposure to sublethal concentrations of NH₃

would cause an increase in oxygen consumption rate in Shawnee rocksnailed and fine-ridged elimia.

Materials and Methods

Fine-ridged elimia were collected from Elkhorn Creek near Frankfort, Kentucky, USA (38°19'11.26"N, 84°49'35.93"W) in the summers of 2016, 2017, and 2018 for righting time experiments and summer 2017 for oxygen consumption experiments. Shawnee rocksnailed were collected from the Rough River near Elizabethtown, Kentucky, USA (37°38'26.5"N, 86°11'52.26"W) in fall 2016, summer 2017, and summer 2018 for righting time experiments and in summer 2017 for oxygen consumption experiments. Species identities of the focal snails were verified by the Museum of Biological Diversity at Ohio State University (catalog # for fine-ridged elimia: OSUM Gastropod 41567, for Shawnee rocksnailed: OSUM Gastropod 42302). Over the course of 2015 to 2018, in the months of April to November, I tested water samples for both creeks approximately every other month to determine the concentration of NH₃.

In the lab, snails were initially housed in water from their source streams for one day, but then were acclimated to reconstituted hard fresh water, which was prepared according to American Public Health Association methods (APHA 2005). This acclimation process took place over the course of at least one week, via daily additions of reconstituted fresh water. While housed in the lab, snails were fed *ad libitum* on TetraMin® tropical flakes (Spectrum Brands, Inc., Blacksburg, VA) and a mixture of diatoms and green algae, including *Oocystis* and *Chlorella*, provided by the Center for Mollusk Conservation in Frankfort, Kentucky, USA. During both experiments, snails were housed individually in 250 mL beakers. Snails were kept at 20°C (±2°C) with a

12:12 light:dark cycle in incubators (Norlake Scientific, Undercounter BOD Refrigerated Incubator). Overall snail size (measured from shell apex to the outer edge of the aperture) was recorded for each individual before the experiments began.

NH₃ solutions were prepared from a stock solution, which was created by dissolving ammonium chloride (NH₄Cl) in reconstituted water (Borgmann 1994; Alonso and Camargo 2009). For the oxygen consumption experiment, stock solutions were made weekly and stored in a refrigerator until needed. The concentration of NH₃ was tested for the stock solution every other day. All testing of NH₃ concentrations for both experiments was done with a spectrophotometer (DR/2000 Direct Reading Spectrophotometer, Hach Company) using the salicylate method, following the protocol of Hach Company (1992). To improve precision and accuracy, absorbance values from the spectrophotometer were used with a standard curve to calculate concentrations of NH₃.

Righting Time Experiments

Average snail sizes (\pm SE) were 11.7 ± 0.07 mm for fine-ridged elimia and 12.0 ± 0.07 mm for Shawnee rocksnails. Each snail's righting time was tested before and after exposure to NH₃ to account for differences in righting time related to size, age, or other individual differences (Fong et al. 2017). Before the pre-exposure righting test, snails were allowed to acclimate to their beakers overnight in 150 mL reconstituted fresh water and were fed *ad libitum* on TetraMin® tropical flakes. Fish flakes were chosen instead of algae as the food source during the experiment because they are easier to portion into individual beakers (Besser et al. 2016).

Pre-exposure tests of righting time were conducted the morning following overnight acclimation. To test righting time, a glass syracuse dish (d = 104 mm, h = 42

mm) was filled with the water from the beaker in which the snail had been housed (water height = 19 mm). The snail was then placed, by hand, on its dorsal surface, with the aperture facing upwards, in the center of the dish, at which point the timer was started. The timer was stopped when the snail had contacted the glass surface with its foot and pulled its shell into the upright position. Snails that required >20 minutes to right themselves in the pre-exposure test were removed from the study.

The day after pre-tests of righting time, solutions of NH₃ were prepared as previously described. Concentrations ranged from 0.25 to 12.51 mg NH₃-N/L for fine-ridged elimia and from 0.34 to 12.61 mg NH₃-N/L for Shawnee rocksnailes. The term NH₃-N is the notation for unionized ammonia (NH₃) concentrations when the molecular weight is based only on the nitrogen component. Up to two concentrations could be tested simultaneously, along with a control group. Thus, I began with concentrations near those reported to affect behavior or survival in comparable studies (Alonso and Camargo 2015; Goudreau et al. 1993) and continued raising the concentration for each species. In total, there were nine rounds of the experiment for fine-ridged elimia and ten for Shawnee rocksnailes, all performed between 2016 and 2018.

Following preparation of the ammonia solutions, each snail's water was changed, either to new control (reconstituted fresh) water (≤ 0.05 mg NH₃-N /L) or to the assigned concentration of NH₃. After 24 hours, righting time was tested again. In post-exposure tests, snails that did not right in 60 minutes were labeled "failed to right." The change in righting time was calculated for each snail by subtracting the pre-exposure righting time from the post-exposure righting time.

Examination of Potential Residual Effects

Due to the unusually high concentrations of NH_3 being tolerated by both species during the 24-hour exposures (see Results), additional tests were performed for fine-ridged elimia in 12.51 mg $\text{NH}_3\text{-N/L}$ and Shawnee rocksnailes in 8.99 mg $\text{NH}_3\text{-N/L}$ and 12.61 mg $\text{NH}_3\text{-N/L}$, as well as the control snails associated with each of those treatment groups. On the same day as the post-exposure righting time tests described above, all treatment and control snails were moved to clean beakers with 150 mL new reconstituted fresh water (i.e. control water), fed, and put back into the incubators. No snails were exposed to NH_3 for the next 10 days. During the 10-day period following NH_3 exposure, the snails were checked daily for survival. Fifty percent water changes were performed every other day, with feeding done on the same day as water changes. On the seventh day of the 10-day post-exposure period, righting time was tested again, using the same methods as the previous post-exposure test. Only one Shawnee rocksnail died during the post-exposure period; it was in the treatment group that had been exposed to 8.99 mg $\text{NH}_3\text{-N/L}$ and it died on the 10th day following ammonia exposure. A single fine-ridged elimia died on the sixth day of the post-exposure period, but it was from the control group.

Oxygen Consumption Experiments

The fine-ridged elimia used in oxygen consumption experiments had an average shell length ($\pm\text{SE}$) of 11.4 ± 0.09 mm and an average wet weight of 0.1667 ± 0.00402 g. Wet weight refers to the entire living snail with the shell attached; it was chosen to avoid killing any snails (Chen et al. 2001). Average Shawnee rocksnail shell length and wet weight were 11.6 ± 0.13 mm and 0.2968 ± 0.00979 g, respectively. The experiment took place in 2017 and involved three separate rounds for each species, each using a new batch

of stock NH_3 solution. Snails were put into their assigned concentration of NH_3 or control reconstituted water (≤ 0.03 mg $\text{NH}_3\text{-N/L}$) on the first day of each round, and oxygen consumption was tested 24 hours later. In the control trials, I tested 40 fine-ridged elimia and 39 Shawnee rocksnailes. Unionized ammonia levels ($\text{NH}_3\text{-N}$) were 10.6 mg/L (fine-ridged elimia: $n=22$; Shawnee rocksnailes: $n=11$) and 15.0 mg/L (fine-ridged elimia: $n=15$; Shawnee rocksnailes: $n=30$). Concentrations varied slightly between replicates (up to 0.6 mg $\text{NH}_3\text{-N/L}$); thus, all values reported are averages. These NH_3 levels were chosen because the lower concentration was near the dose that affected righting behavior for fine-ridged elimia in the righting time tests.

At the beginning of each round, the snails were weighed, in addition to being measured, for later calculations of mass specific oxygen consumption rates. The snails' shells were dried before weighing and individuals from the control and treatment groups were approximately size-matched. The snails were put individually into beakers with 150 mL of their assigned treatment, or reconstituted fresh water for controls.

During the 24 hours of exposure to NH_3 (or control water), all snails were kept in incubators as described above. Afterwards, oxygen consumption was assessed. First, the snails' shells were cleaned with a Kimwipe® to remove algae and bacteria (Alexander and McMahon 2004; Hahn 2005). Following the methods of Alexander and McMahon (2004), a respirometer (YSI 5300A Biological Oxygen Monitor) was used to determine oxygen consumption, accounting for oxygen consumption associated with microbial activity by using a second chamber containing only water as a control (i.e. blank). A water bath surrounding the chambers maintained constant temperature at 20°C. Oxygen consumption was calculated for each snail by determining the amount of oxygen used per

hour for the blank and subtracting that from the same value for the snail. This value was then converted into the oxygen consumption rate as $\mu\text{g O}_2 \cdot \text{hr}^{-1} \cdot \text{g wet wt}^{-1}$. Snails were tested for oxygen consumption in water without added NH_3 , regardless of their treatment group, in order to maintain proper functioning of the oxygen probe (Cheung and Wong 1998). Additionally, prior to testing oxygen consumption, the water in which the snail will reside in the respirometer must be aerated to nearly 100% saturation, which would drive off volatile chemicals such as NH_3 .

Statistical Analyses

All statistical tests were conducted in the R platform (ver. 3.3.1) (R Core Team 2016). For each species, linear regression with permutation tests was used to test for an effect of NH_3 concentration on change in righting time (lmPerm package, Wheeler and Torchiano 2016). Permutation tests were used because the change in righting time was not normally distributed and transformations could not correct normality (Kabacoff 2015). Shell length was included in the model as a covariate; length was used to represent the snail size. Experimental round was also included in the model as a random factor (though the designation is not necessary with permutation tests). Logistic regression was used to test whether the concentration of ammonia affected the proportion of snails that failed to right in the post-exposure test. Where a significant effect was found, Dunnett's test was used to determine which treatment groups differed from the control group. Data from the residual effects tests were analyzed in the same manner as above, but with change in righting time representing the difference in righting time seven days after ammonia exposure and before ammonia exposure.

For each species, mixed effect analysis of covariance (lmerTest package, Kuznetsova et al. 2016) was used to test for an effect of the concentration of NH₃ on the rate of oxygen consumption, while controlling for the effects of snail size (both shell length and gross wet weight). The models for each species included experimental round as a random effect.

Results

Over the course of 2015-2018 (in the months of April to November), I recorded an average concentration (\pm SE) of 0.025 ± 0.010 mg NH₃-N/L for Elkhorn Creek and 0.053 ± 0.016 mg NH₃-N/L for the Rough River; maximum concentrations were 0.056 mg NH₃-N/L for Elkhorn Creek and 0.178 mg NH₃-N/L for the Rough River.

Righting Time Experiments

Sample sizes, pre-exposure righting time averages, and post-exposure righting time averages for each species at each treatment level can be found in Table 1 in Appendix A. Sample sizes differed among treatment groups due to variation in the number of snails that righted within the 20-minute time limit for pre-exposure tests. Overall, 4.4% of fine-ridged elimia were removed from the study for failing to right within 20 minutes in pre-exposure tests; 55.4% of Shawnee rocksnailed were removed for the same reason.

Neither species' change in righting time was affected by the random effect, experimental round (fine-ridged elimia: $p=0.667$; Shawnee rocksnailed: $p=0.278$); thus it was dropped from both models. Acute exposure to NH₃ affected righting behavior for both species tested in this study, but in different ways. NH₃ affected the change in righting time for fine-ridged elimia ($p<0.001$), with snails exposed to higher concentrations of NH₃ righting more slowly after the 24-hour exposure period (Figure 1).

However, control snail change in righting time only differed significantly from that of snails exposed to 9.03 mg NH₃-N/L; snails in this treatment group righted 2.48 min more slowly on average in post-exposure tests, as compared to a decrease in righting time of 0.35 min for controls (p=0.021). Shawnee rocksnail change in righting time was not affected by ammonia concentration (p=0.882) but it was affected by shell length (p=0.017). Larger Shawnee rocksnails righted slightly more slowly after the exposure period than smaller snails; however, size explained very little of the variation in change in righting time (R² =0.01). Shell length did not affect change in righting time for fine-ridged elimia (p=1).

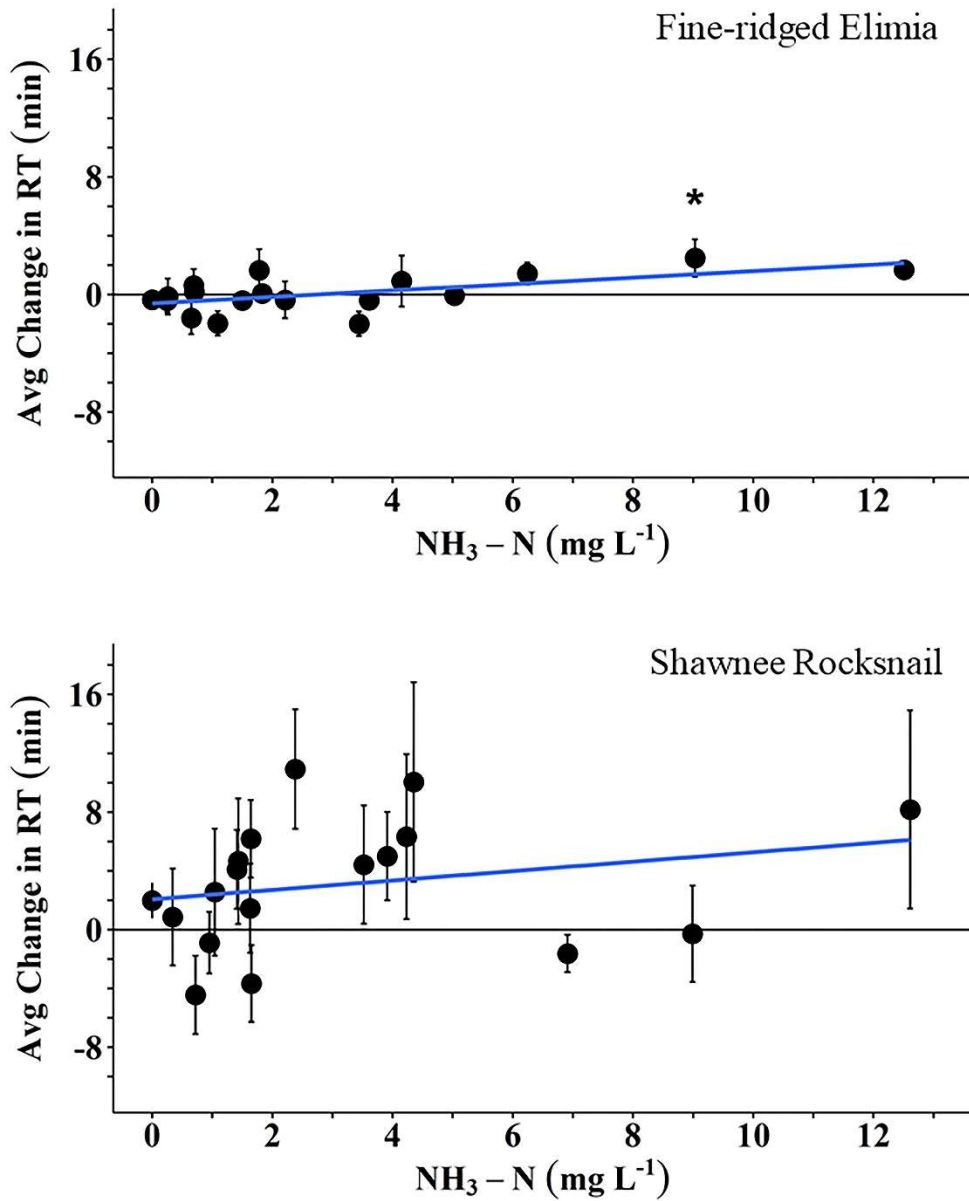


Figure 1. Average change in righting time (RT) for snails exposed to unionized ammonia (NH₃) for 24 hours. Positive values for change in righting time indicate righting more slowly in post-exposure tests; negative values indicate righting more quickly after the exposure period. Bars represent standard error. Treatment groups that differ significantly from the control group (≤ 0.05 mg NH₃-N/L) are indicated by an asterisk.

While NH₃ did not cause Shawnee rocksnails to right more slowly in the range of tested doses, it negatively impacted righting success ($p=0.0046$, $\chi^2=8.048$, $df=1$; Figure 2); 61.5% of snails exposed to 12.61 mg NH₃-N/L failed to right themselves in post-exposure tests, as compared to 18.5% of snails in control conditions ($p=0.004$). NH₃ did not affect righting success for fine-ridged elimia ($p=0.103$, $\chi^2=2.667$, $df=1$).

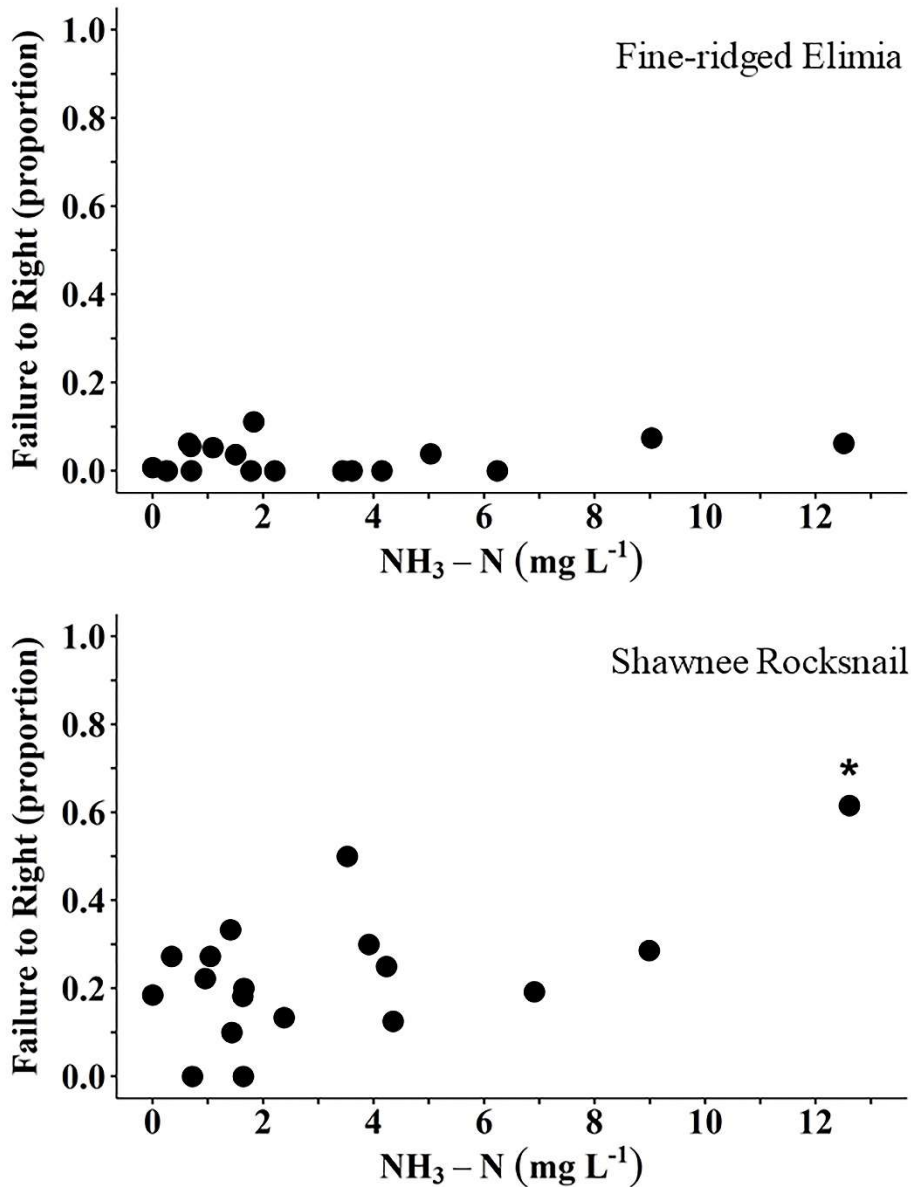


Figure 2. Proportion of snails that failed to right (within 60 minutes) after 24-hour exposure to unionized ammonia (NH₃). Treatment groups that differ significantly from the control group (≤ 0.05 mg NH₃-N/L) are indicated by an asterisk.

Tests for Residual Effects

Results from residual effects tests suggest that fine-ridged elimia and Shawnee rocksnails recover from exposure to NH₃ within seven days of returning to regular water. The

concentration of NH₃ applied seven days prior did not affect change in righting time for either species (fine-ridged elimia: p=0.902; Shawnee rocksnail: p=0.106; Figure 3). Additionally, there was no effect of snail size (fine-ridged elimia: p=0.961; Shawnee rocksnail: p=0.077) or experimental round (Shawnee rocksnail: p=0.540; fine-ridged elimia: not applicable) on change in righting time for the residual effects tests. The concentration of NH₃ applied seven days prior did not affect the proportion of snails that failed to right (fine-ridged elimia: p=0.100, $\chi^2 = 2.71$, df=1; Shawnee rocksnail: p=0.236, $\chi^2 = 1.41$, df=1; Figure 3).

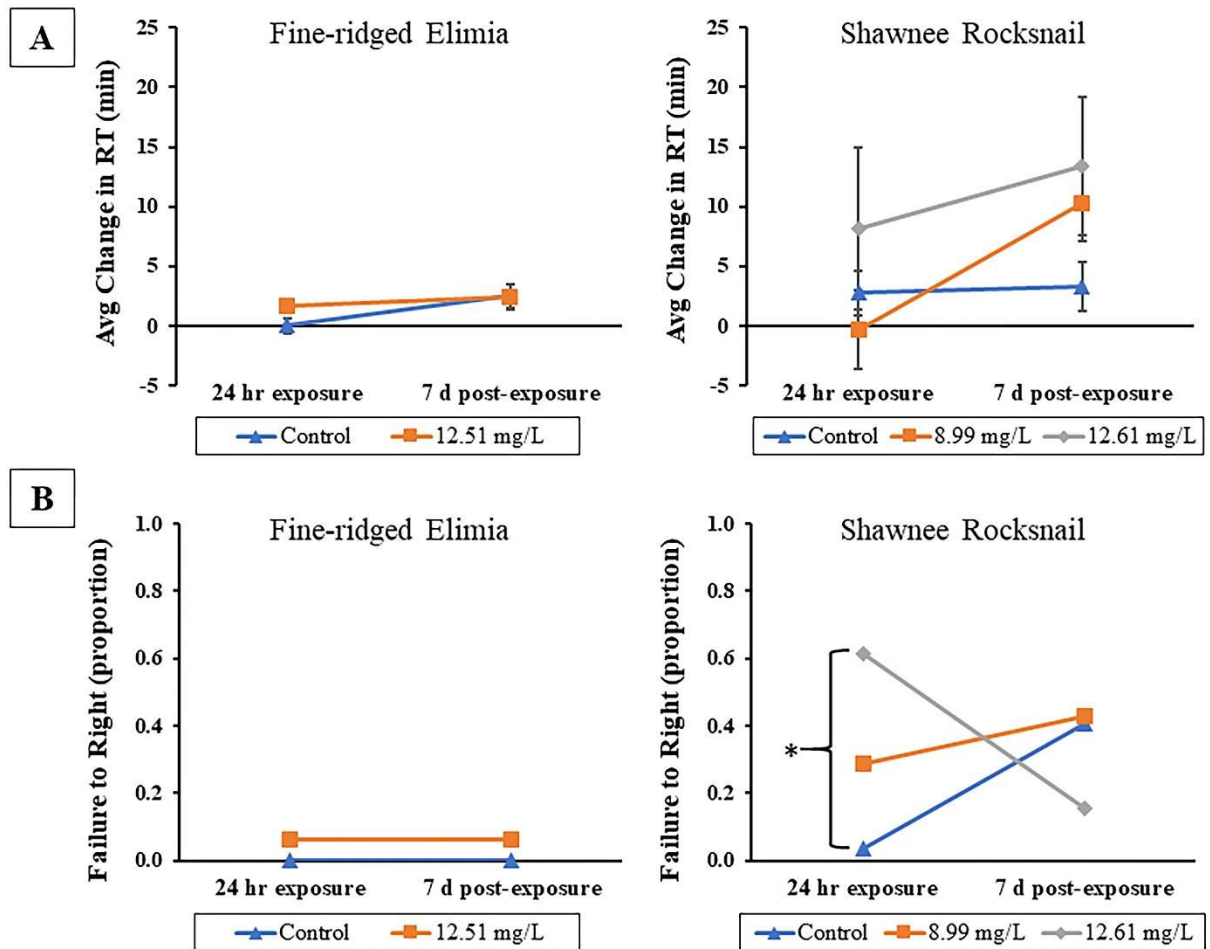


Figure 3. Average change in righting time and proportion of snails that failed to right after 24-hour exposure to unionized ammonia (NH_3) and seven days after exposure to NH_3 ended. Bars represent standard error. Significant differences ($p < 0.05$) between groups are indicated with an asterisk. All concentrations represent mg $\text{NH}_3\text{-N/L}$. Control solutions contained ≤ 0.05 mg $\text{NH}_3\text{-N/L}$. (A) Change in righting time refers to the righting time at a given time point minus the pre-exposure righting time. (B) Failure to right refers to failing within 60 minutes.

Oxygen Consumption Experiments

Little to no respiration occurred in the blanks ($<7.92 \mu\text{g O}_2/\text{hr}$). Acute exposure to NH_3 did not affect the rate of oxygen consumption for either species at any concentration tested (fine-ridged elimia: $p=0.364$, $t=-0.92$, $df=73$; Shawnee rocksnail: $p=0.857$, $t=-0.18$, $df=74$; Figure 4). Neither species displayed an effect of shell length on oxygen consumption (fine-ridged elimia: $p=0.530$, $t=1.97$, $df=73$; Shawnee rocksnail: $p=0.219$, $t=1.24$, $df=74$). Snail wet weight did not affect the rate of oxygen consumption for Shawnee rocksnails ($p=0.069$, $t=-1.85$, $df=74$), but it did negatively affect the rate of oxygen consumption for fine-ridged elimia ($p=0.015$, $t=-2.50$, $df=73$). Larger fine-ridged elimia had slightly lower rates of oxygen consumption, but weight explained only 4% of the variation in oxygen consumption ($R^2=0.04$).

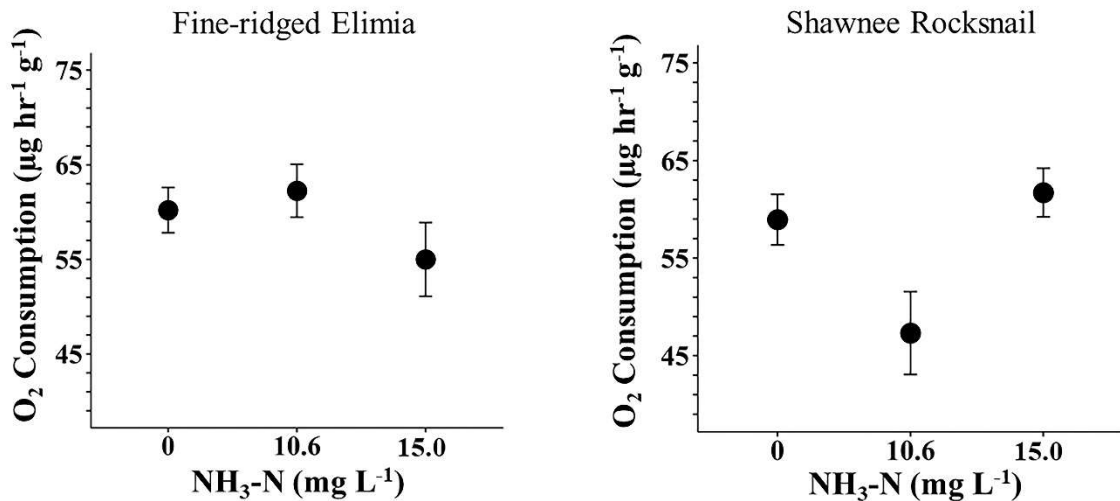


Figure 4. Average rate of oxygen consumption after 24-hour exposure to unionized ammonia (NH_3). Bars represent standard error. Concentrations of NH_3 are averages of actual concentrations, which ranged from 10.5-10.6 and 14.7-15.3 mg $\text{NH}_3\text{-N/L}$.

Discussion

Ammonia pollution has the potential to confer serious negative effects on freshwater biodiversity via disruption of animal behavior and physiology (e.g. Muñoz et al. 2012; Schalkhausser et al. 2014; Zhang et al. 2017). Despite increasing evidence for such consequences, this study shows that natural populations of fine-ridged elimia and Shawnee rocksnails are unaffected by typical ammonia levels and are able to recover from exposure to extremely high pulses of ammonia. While the tolerances of these two species differ slightly, both appear to behave and respire normally when exposed to doses of NH_3 that are considered lethal to other aquatic taxa.

There are several implications of the impacts of high doses of NH_3 on fine-ridged elimia. Righting more slowly costs snails feeding time (Wei et al. 2016) and could potentially increase exposure to predators. Snails typically right more quickly when predator scent is present (Orr et al. 2007), but this adaptation could be lost or impaired when righting behavior is slowed by exposure to ammonia. Furthermore, these effects are expected to be more severe for Shawnee rocksnails when they are exposed to high doses of ammonia, as 61.5% of them failed to right themselves at all (compared to 18.5% in control conditions).

While the effects of righting slowly or failing to right can be problematic for snails, these effects are unlikely to be seen in natural populations of Shawnee rocksnails and fine-ridged elimia. First, fine-ridged elimia only experienced a maximum average increase in righting time of 2.48 minutes in this study (with exposure to 9.03 mg NH_3 -N/L), as compared to -0.35 minutes on average for controls. Shawnee rocksnails experienced more severe impairments of righting behavior with increased failure to right

at 12.61 mg NH₃-N/L, but regardless, the doses of ammonia required to elicit behavioral effects for both species are well above the concentrations of NH₃ in the snails' source streams. Over the course of 2016 to 2018, I recorded a maximum value of 0.056 mg NH₃-N/L for Elkhorn Creek, where fine-ridged elimia were collected, and a maximum value of 0.178 mg NH₃-N/L for the Rough River, where Shawnee rocksnailed were collected. Thus, it is extremely unlikely that the populations of snails used in these experiments are currently experiencing impairment of righting behavior in their natural habitats due to NH₃ exposure.

Additionally, my results suggest that fine-ridged elimia and Shawnee rocksnailed recover from exposure to NH₃ within seven days of returning to water containing no NH₃, suggesting that there are no lasting effects after a pulse event ends and the NH₃ is flushed downstream. At the highest concentrations tested, neither species righted significantly more slowly than controls seven days after the 24-hour exposure period, nor did the proportion failing to right differ from controls. The freshwater mussel (*Anodonta anatina*) similarly returns to normal behavior within seven days of exposure to de-icing salt (Hartmann et al. 2016). Freshwater planaria (*Polycelis felina*) also recover behaviorally within seven days of exposure to NH₃, but after two additional pulses of ammonia, they fail to fully recover in the same amount of time (Alonso and Camargo 2015). Shawnee rocksnailed and fine-ridged elimia may not recover their righting abilities as well as they did in this study if they were exposed to multiple pulses of NH₃, an approximation of recurring rainstorms or other ammonia-releasing events (Yahdjian and Sala 2010).

Regardless of whether behavioral impairments caused by high doses of ammonia last longer with additional pulses of the toxin, these snails' tolerances to NH₃ are extremely high compared to other taxa. Locomotion behavior is significantly impaired at 0.20 mg NH₃-N/L for freshwater planaria (Alonso and Camargo 2015) and at 0.07 mg NH₃-N/L for New Zealand mudsnails (*Potamopyrgus antipodarum*, Alonso and Camargo 2009), though both studies used exposure periods ≥ 10 days. Even lethal concentrations for other species are well below the 12.61 mg NH₃-N/L it took to affect righting success for Shawnee rocksnailed and the 9.03 mg NH₃-N/L it took to affect righting time for fine-ridged elimia in this study. The 24 hr LC₅₀ for New Zealand mudsnails is 2.72 mg NH₃-N/L (Alonso and Camargo 2003) and the 96 hr LC₅₀ for the freshwater snail *Pleurocera uncialis uncialis* is 0.742 mg NH₃-N/L (Goudreau et al. 1993). For comparison, the 96 hr LC₅₀ for rainbow trout (*Oncorhynchus mykiss*) is 0.296 mg NH₃-N/L (Thurston et al. 1981).

In the future, it would be beneficial to study the impacts of NH₃ on different life stages. This study and many others focused only on adult snails, but it is possible that juvenile snails experience behavioral impacts at lower concentrations than adults. This pattern has been documented for mortality in many invertebrates with exposure to various chemicals (e.g. Aguirre-Sierra et al. 2011; An et al. 2013; Archambault et al. 2015). Clearly juvenile survival and fitness are important to the conservation of snail species; thus, it is important to determine how environmental stressors like NH₃ affect juveniles as well.

There remains uncertainty surrounding the underlying reason righting behavior is affected by NH₃ (albeit very high doses). Doses up to 6 mg NH₃-N/L greater than the

doses that affected behavior still had no significant effect on the snails' oxygen consumption rates. This result is not completely surprising based on zebrafish toxicological research. Behavioral impairments occur at much lower concentrations of the fungicide carbendazim than it takes to affect metabolism for early life-stage zebrafish (*Danio rerio*, Andrade et al. 2016). Thus, oxygen consumption in snails could be affected by higher doses of NH_3 than were tested in this study, but ultimately, the behavioral impairments seen in this study are unlikely to have been caused by respiratory damage or metabolic stress. Impairments of behavior caused by exposure to toxins are usually signs of underlying physiological issues (Pyle and Ford 2017). If the physiological issue causing impairment of righting behavior for Shawnee rocksnails and fine-ridged elimia is not respiratory, perhaps it is related to the central nervous system, as has been suggested to explain effects of ammonia on fish, such as increases in gill ventilation and convulsions (Russo 1985).

There is still a great deal to learn about the sublethal impacts of NH_3 . This research highlights the incredible breadth of gastropod tolerance to NH_3 , as well as the lack of clarity regarding the physiological causes of behavioral impairments induced by this chemical. Additional comparative studies are needed to reach the Freshwater Mollusk Conservation Society's goal of determining similarities of sensitivities across species and genera (FMCS 2016). The results of this study suggest that ammonia pollution poses very little risk to natural populations of Shawnee rocksnails and fine-ridged elimia, but the massive difference in these species' tolerances and those of the other gastropods tested so far highlights the utmost importance of studying more species rather than relying on generalizations based on one or a few.

CHAPTER II
IMPACTS OF ELEVATED WATER TEMPERATURES ON RIGHTING BEHAVIOR
AND SURVIVAL OF TWO FRESHWATER CAENOGASTROPOD SNAILS

Introduction

With surface temperatures on Earth increasing at a rate of 0.05°C per decade, and the ocean surface warming even faster (0.11°C per decade), efforts to understand the effects of climate warming have likewise been increasing (IPCC 2014). Streams and rivers are experiencing greater increases in average temperature than oceans and may be more sensitive to climate change than any other ecosystem (Durance and Ormerod 2009; Isaak et al. 2012). One reason for the expected severity of effects to freshwater communities is limitations to movement (Shuter and Post 1990). Animals in freshwaters often cannot move to cooler waters because of physical barriers or the difficulty of moving upstream for taxa with limited mobility; thus, extinction rates and extirpations of freshwater taxa have so far matched or surpassed those of terrestrial species (Heino et al. 2009). In general, freshwater snails can actively move upstream by <1.0 km per year (Kappes and Haase 2012). As such, predictions show that active dispersal is not likely to be fast enough for freshwater mollusks to compensate for the predicted rate of climate warming (Kappes and Haase 2012; Alexander and Wagoner 2016).

In addition to limitations on movement, the ectothermic nature of aquatic invertebrates puts them at the mercy of the environment. Freshwater mussels have

already experienced reduced diversity and density (Galbraith et al. 2010) and thermophilic invertebrates have been incrementally replacing upstream, northern species (Daufresne et al. 2004). While temperatures rising above thermal limits can result in movement of some species and population extinctions for others, small temperature changes can have large impacts on freshwater communities as well. Elevated environmental temperatures increase metabolism and respiratory rates in most ectothermic organisms by eliciting greater oxygen demand (Schalkhausser et al. 2014; Zhang et al. 2014; Alexander and Wagoner 2016). Many behaviors are impacted by elevated temperatures as well, such as lowered reproductive output and advanced timing of spawning (Philippart et al. 2003), reduced activity levels (Verdelhos et al. 2015), increased burrowing (Clements et al. 2017), reduced duration of escape response (Schalkhausser et al. 2014), and increased swimming speeds (Zhang et al. 2014).

When diverse animals like snails, turtles, sea stars, crayfish, and sea urchins find themselves flipped onto their dorsal sides, the attempt to turn the body right-side-up is called righting behavior. This behavior has been shown to be impacted by climate change, but few species have been tested so far (Antarctic limpet (*Nacella concinna*), Peck et al. 2004; land snail (*Cornu aspersum*), Gaitán-Espitia et al. 2013; Antarctic marine snail (*Margarella antarctica*), Schram et al. 2014; sea urchin (*Lytechinus variegatus*), Brothers and McClintock 2015). While in the upside-down position, snails in particular may be vulnerable to predators as their aperture is exposed; evidence for this idea arises from research showing that escape and righting behaviors are induced and/or accelerated by the presence of predators (Lemmnitz et al. 1989; Orr et al. 2007). Furthermore, with the body in this position, normal activities, such as feeding and

mating, cannot be accomplished (Wei et al. 2016). Thus, increased righting time is used as an indicator of stress in gastropods and other aquatic groups (e.g. Burger 1998; Fong et al. 2017; Zhang et al. 2017).

Righting behavior may become increasingly important for the success of snails living near shore lines because the combination of altered rainfall patterns and periodic increased evaporation associated with climate warming results in greater water level fluctuations (Heino et al. 2009). These fluctuations can result in exposure of snails to drying, and while at least one species is known to respond to decreasing water levels by moving to deeper water or burrowing into the sediments (Poznańska et al. 2015), this adaptation could be compromised if warming temperatures impair the snails' ability to right themselves.

While several studies have found effects of elevated temperature on righting behavior for various aquatic taxa (as previously discussed), few studies of this kind have focused on temperate or freshwater snails (e.g. invasive freshwater snail (*Potamopyrgus antipodarum*), Sharbrough et al. 2017). Snails are currently in a precarious state, with 74% of North American species listed as imperiled (vulnerable, threatened, endangered) or extinct as of 2013 (Johnson et al. 2013). Freshwater snails play several important roles in their ecosystem; they accelerate the decomposition process of leaf litter (Chadwick et al. 2006) and control the biomass of periphyton, which covers submerged surfaces (Hill 1992; Brown et al. 2008). Based on the perilous state of snail diversity and the importance of their presence in freshwater habitats (along with mussels), an urgent call has been made for further research on the sublethal impacts of environmental stressors with regards to mollusks (FMCS 2016).

In order to increase our understanding of the potential impacts of elevated temperatures associated with climate warming to freshwater snails, I tested whether chronic exposure to elevated temperatures affects righting time for fine-ridged elimia (*Elimia semicarinata*, Say 1829) and Shawnee rocksnails (*Lithasia obovata*, Say 1829) (both Gastropoda: Neotaenioglossa: Pleuroceridae). It should be noted that recent research has suggested that *Lithasia obovata* may be a synonym for *Pleurocera semicarinata* (Dillon 2014) and the genera *Elimia* and *Pleurocera* may also be synonymous (Dillon 2011). However, due to the ongoing transitional state of pleurocerid taxonomy, I retain the traditional taxonomy for the time being, following Johnson et al. (2013). Fine-ridged elimia are found in Kentucky, Indiana, and Ohio, USA; Shawnee rocksnails extend beyond these states into Illinois, Pennsylvania, and Tennessee, USA (Johnson et al. 2013). Both species are generally found in shallow, slow-flowing portions of streams and rivers, largely on rocks and logs where they graze on periphyton (Johnson and Brown 1997, Greenwood and Thorp 2001). I chose to study these species because they are locally abundant in Kentucky, and therefore, removing several hundred individuals should not negatively impact their populations. I hypothesized that elevated, sublethal temperatures would have a negative effect on snail righting time, with snails righting more slowly at higher temperatures.

Materials and Methods

Snail collection occurred in 2017 August for fine-ridged elimia and 2017 September-October for Shawnee rocksnails. Both species have been verified by the Museum of Biological Diversity at Ohio State University (catalog number for fine-ridged elimia: OSUM Gastropod 41567, for Shawnee rocksnails: OSUM Gastropod 42302). Fine-ridged

elimia were collected from Elkhorn Creek near Frankfort, Kentucky, USA (38°19'11.26"N, 84°49'35.93"W) and Shawnee rocksnails were collected from the Rough River near Elizabethtown, Kentucky, USA (37°38'26.5"N, 86°11'52.26"W). The average daily water temperature of Elkhorn Creek in summer 2016 was 28.1°C; the maximum temperature for the entire year was 34.3°C (USGS 2017a). In the Rough River, average daily summer temperature was 19.9°C in 2016 and the maximum temperature was 28.6°C (USGS 2017b; see Figure 1 in Appendix B for year-long stream temperature data).

Once in the lab, all snails were housed in aquaria with water from their home streams. Over the course of at least one week, aquarium water was incrementally changed to reconstituted hard fresh water, which was prepared according to American Public Health Association methods (APHA 2005). During this acclimation time, snails were fed ad libitum on a mixture of green algae and diatoms, provided by the Center for Mollusk Conservation in Frankfort, Kentucky, USA.

The experiment was run in several rounds for each species: two rounds for fine-ridged elimia, in 2017 August-September and three rounds in 2017 September-November for Shawnee rocksnails. A control group (20°C) was tested in each round along with the elevated temperature treatment. A third round was performed for Shawnee rocksnails to increase the sample size of snails exposed to 27°C for greater statistical power. Throughout the experiment, snails were housed in individual 250 mL beakers containing 150 mL reconstituted hard fresh water. These beakers were kept in incubators (Norlake Scientific, Undercounter BOD Refrigerated Incubator) with 12/12 light/dark cycles. On the first day of the experiment, snails were allowed to acclimate to their beakers in

incubators at 20°C overnight. The next day, righting time was tested for each snail by placing the snail on its dorsal surface, with the aperture facing up, in a Syracuse dish (d= 10.4 cm, h= 4.2 cm) with all of the water from its beaker (water depth = 1.9 cm). To reduce variation in the test conditions, no substratum was used. These pre-tests were done at room temperature (20°C) to provide a baseline righting time for each snail before the treatment groups were exposed to elevated temperatures. Pre-tests also helped to account for potential differences in righting time related to size, age, sex, or other factors (Fong et al. 2017). However, age was controlled to some degree as only adult snails were included in this study. The amount of time it took the snail to reach the glass surface with its foot and pull the shell back to its proper place was recorded as the snail's righting time. Fine-ridged elimia individuals were removed from the study if they did not right within 20 minutes; Shawnee rocksnailes were removed if they did not right within 30 minutes. This time limit helped to ensure that only healthy snails were included in the study. Shawnee rocksnailes were given additional time compared to fine-ridged elimia because preliminary tests revealed that they generally right more slowly, even when unimpeded by outside influences.

After righting time pre-tests, all snails were randomly assigned to treatment groups by size (working from smallest to largest shell length). One incubator was maintained at 20°C to serve as a control (n= 34 Shawnee rocksnailes, 40 fine-ridged elimia). Treatment temperatures for fine-ridged elimia were 30°C (n=22) and 35°C (n=40); for Shawnee rocksnailes they were 27°C (n=25) and 30°C (n=11). We originally planned to test all snails at 30°C and 35°C, but high mortality for fine-ridged elimia at 35°C prompted us to reduce the temperature treatments for Shawnee rocksnailes. The non-

control incubators were incrementally brought to the assigned temperature over the course of two days. Only one temperature was tested against a control group in each round of the experiment.

Snails were kept at the elevated temperatures for 10 days, making this a chronic test (Brothers and McClintock 2015, Clements et al. 2017). Every other day, half the water in each beaker was changed using water pre-heated to the snail's assigned temperature and all snails were fed TetraMin® tropical flakes (www.tetra-fish.com, Spectrum Brands, Inc., Blacksburg, VA) ad libitum. Dissolved oxygen and pH were tested on alternate days from water changes; the samples were arbitrarily selected from two snail beakers per treatment group. Temperature was recorded for each incubator daily. On the tenth day of the experiment, righting time was tested again. The control group was tested in the same manner as before. Righting time for the elevated temperature groups was tested using a warm water bath to maintain assigned temperatures. An aquarium was filled with warm water and 6 glass dishes were submerged; the snails' Syracuse dishes were placed on top of the submerged dishes such that the water in the snail's dish would not mix with the water bath surrounding it. The water baths kept the snails' water within 2°C of the assigned temperature, although there were a few exceptions in which the temperature reached 3 to 4°C above the assigned temperature for a portion of the behavior test. These exceptions only occurred for Shawnee rocksnaileds in the 27°C treatment group. For all treatment groups, if snails did not right themselves in a maximum amount of time (60 minutes for fine-ridged elimia, 90 minutes for Shawnee rocksnaileds), they were recorded as failing to right.

All statistical tests were conducted in the R platform (ver. 3.3.1) (R Core Team 2016) and were performed separately for the two species. (Approximate) exact logistic regression (elrm package; Zamar et al. 2007) was used to test for an effect of temperature on snail mortality as proportions of total numbers of snails tested. MCMC sampling parameters were set to 1000 iterations with 0 burnin. This test was chosen because instances of low or zero counts, which were present in several treatment groups for both species, can be problematic for regular logistic regression (UCLA 2018). Logistic regression was used to test for an effect of temperature on the proportion of snails that failed to right in the post-exposure test. The uppermost temperature treatment groups (35°C for fine-ridged elimia, 30°C for Shawnee rocksnails) were omitted from this analysis, as well as analyses of righting time, because high mortality resulted in small sample sizes for post-exposure righting time tests.

Pre-exposure righting time values were subtracted from post-exposure values to obtain the change in righting time for each snail. Positive values indicate that snails right more slowly after spending ten days in a given temperature; negative values indicate faster righting after the chronic exposure. The effect of temperature on change in righting time was determined for each species using ANCOVA with permutation tests (lmPerm package, Wheeler and Torchiano 2016), because the data were not normally distributed and normality could not be corrected with transformations (Kabacoff 2015). The permutation ANCOVA included snail shell length (from apex to outer edge of aperture) and gross wet weight (combined shell and body weight) as covariates. Additionally, experimental round was included in the model as a random effect variable for Shawnee rocksnails because the 27°C treatment was tested in two rounds.

Results

Incubator temperatures and pH and dissolved oxygen values from the 10-day exposure period are listed in Table 1 for both species. Fine-ridged elimia shell lengths ranged from 9.1 mm to 13.9 mm; gross weights ranged from 0.09 g to 0.30 g. Shawnee rocksnail shell lengths ranged from 9.0 mm to 14.2 mm and gross weights ranged from 0.11 g to 0.48 g.

Table 1. Average water quality data from snail beakers over the 10-day exposure period.

fine-ridged elimia (<i>Elimia semicarinata</i>)		
Incubator Temperature (\pm SE)	pH ^a (\pm SE)	Dissolved Oxygen ^a (\pm SE)
20.4 \pm 0.2°C	7.75 \pm 0.03	6.6 \pm 0.2 mg O ₂ · L ⁻¹
30.4 \pm 0.1°C	7.61 \pm 0.04	4.2 \pm 0.2 mg O ₂ · L ⁻¹
35.0 \pm 0.2°C	7.79 \pm 0.01	5.2 \pm 0.8 mg O ₂ · L ⁻¹

Shawnee rocksnail (<i>Lithasia obovata</i>)		
Incubator Temperature (\pm SE)	pH (\pm SE)	Dissolved Oxygen (\pm SE)
20.4 \pm 0.1°C	7.68 \pm 0.05	6.1 \pm 0.2 mg O ₂ · L ⁻¹
26.4 \pm 0.2°C	7.58 \pm 0.02	4.5 \pm 0.1 mg O ₂ · L ⁻¹
30.0 \pm 0.1°C	7.58 \pm 0.04	3.4 \pm 0.4 mg O ₂ · L ⁻¹

^a Each pH and dissolved oxygen measurement was taken from two arbitrarily selected beakers per incubator.

Survival was significantly impacted by temperature for both species (Figure 1). Fine-ridged elimia experienced 95% mortality at 35°C (38 snails out of 40), as compared with 0% mortality at 20°C ($p=0.001\pm 0.001$). P-values for (approximate) exact logistic regression are reported with Monte Carlo standard error values (UCLA 2018). While 9% of the snails exposed to 30°C (2 of 22 snails) died during the study, the difference in mortality between this group and the control group was not significant ($p=0.105\pm 0.005$). A very similar pattern was seen in Shawnee rocksnails, but at lower temperatures. The

control group experienced 8.8% mortality (3 snails out of 34), which was not significantly different from the 8.0% mortality of the 27°C group (2 snails out of 25; $p=1.0\pm 0.0$), but which was significantly lower than the 72.7% mortality of the 30°C group (8 snails out of 11; $p=0.001\pm 0.001$).

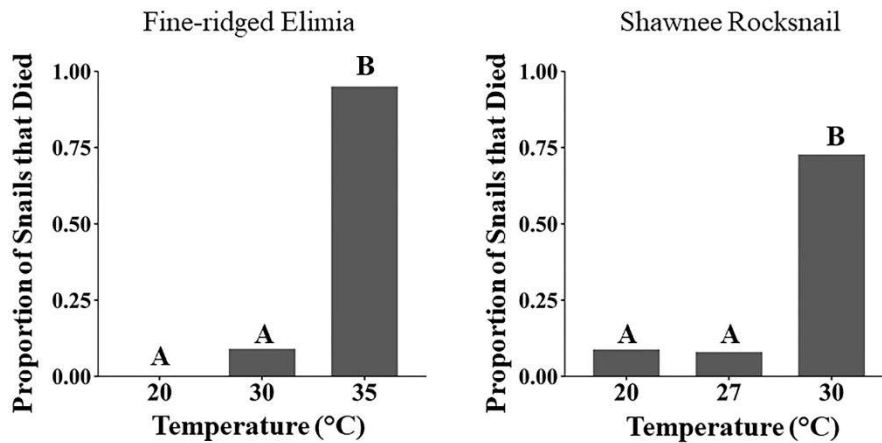


Figure 1. Proportion of snails that died during the 10-day exposure period to various temperatures. Different letters represent significant differences in proportions.

There were significant differences in failure to right across the temperatures for each species (Figure 2). For fine-ridged elimia, only 1 snail out of 40, or 2.5%, of control snails failed to right themselves in the post-exposure test, compared to 30% (6 snails out of 20 that survived to the post-exposure test) in the 30°C group ($p=0.002$, $\chi^2=9.44$, $df=1$). Failure to right for the Shawnee rocksnail control group was higher at 19.4% (6 snails out of 31), but there was still a significant increase in failure to right for snails exposed to elevated temperatures. Of the 27°C group, 11 snails out of 23, or 47.8%, failed to right ($p=0.026$, $\chi^2=4.97$, $df=1$).

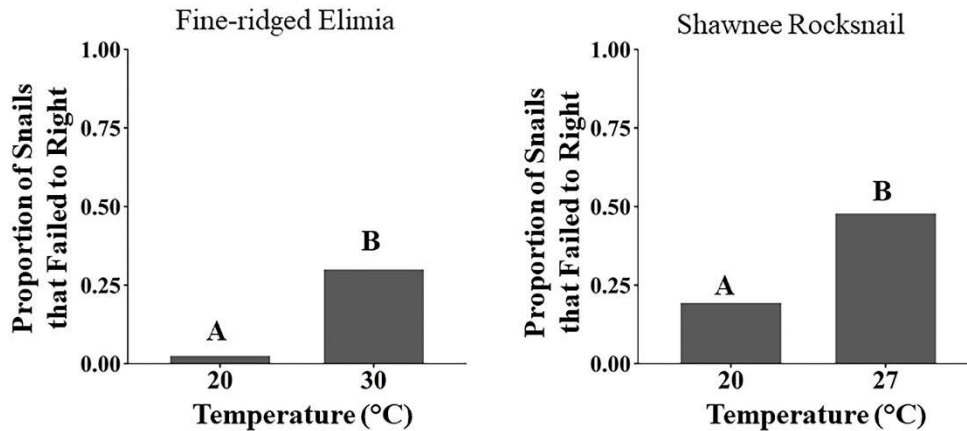


Figure 2. Proportion of snails that failed to right themselves within 60 minutes for fine-ridged elimia (*Elimia semicarinata*) and 90 minutes for Shawnee rocksnails (*Lithasia obovata*) after 10-day exposure to different temperatures. Different letters represent significant differences in proportions. The uppermost temperatures tested (35°C for fine-ridged elimia, 30°C for Shawnee rocksnails) are not included because too few snails survived to the post-exposure test to be included.

Neither species displayed a significant effect of temperature on the change in righting time (fine-ridged elimia: $p=0.353$, $n= 39$ and 14 for 20°C and 30°C respectively; Shawnee rocksnail: $p=0.382$, $n= 25$ and 12 for 20°C and 27°C respectively; Figure 3). Average righting times from pre and post-exposure tests are provided in Table 2. Neither species showed a significant relationship between shell length and change in righting time or gross snail weight and change in righting time. For Shawnee rocksnails, change in righting time did not differ significantly between the two 27°C experimental rounds.

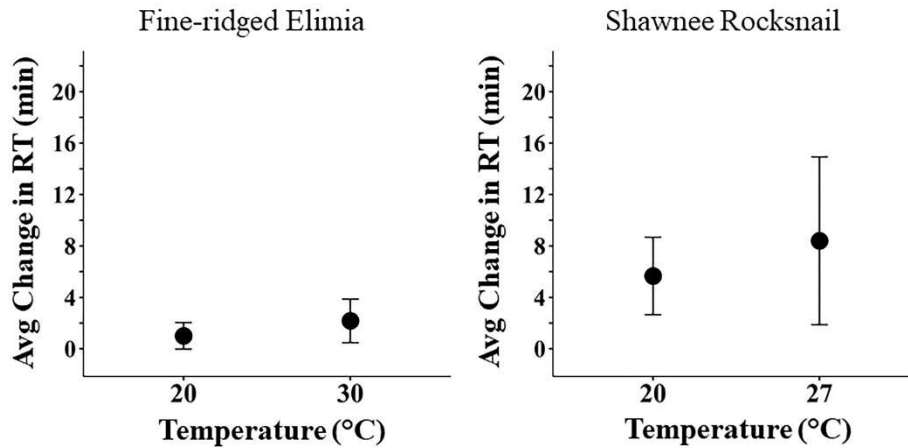


Figure 3. Average change in righting time (RT) for snails chronically exposed to different temperatures. Error bars represent standard error. The uppermost temperatures tested (35°C for fine-ridged elimia (*Elimia semicarinata*), 30°C for Shawnee rocksnails (*Lithasia obovata*)) are not included because too few snails survived to the post-exposure test to be included.

Table 2. Average righting time (RT) for temperature treatment groups of snails in pre-exposure tests and post-exposure tests (following 10-day exposure to assigned temperatures).

fine-ridged elimia (<i>Elimia semicarinata</i>)		
Temperature Treatment	Pre-exposure Test Avg RT (\pm SE)	Post-exposure Test Avg RT (\pm SE)
20°C	5.06 \pm 0.56 min	6.07 \pm 0.84 min
30°C	4.62 \pm 0.61 min	6.80 \pm 1.60 min

Shawnee rocksnail (<i>Lithasia obovata</i>)		
Temperature Treatment	Pre-exposure Test Avg RT (\pm SE)	Post-exposure Test Avg RT (\pm SE)
20°C	11.91 \pm 1.68 min	17.58 \pm 2.74 min
27°C	7.99 \pm 2.05 min	16.39 \pm 5.84 min

Discussion

This study provides evidence that two species of freshwater snails are sensitive to elevated temperatures not far above those currently experienced by natural populations in summer. At the highest temperatures tested, survival was significantly reduced for both species (see Figure 1). Within ten days of exposure to constant 35°C, fine-ridged elimia experienced 95% mortality and in the same time frame at 30°C, Shawnee rocksnails experienced 72.7% mortality. These high mortality rates are troubling for future temperature increases in these species' habitats, because the streams already reach maximum summer temperatures near or above these lethal temperatures. Elkhorn Creek, where fine-ridged elimia were collected, reached a maximum temperature of 34.3°C in 2016 (USGS 2017a); the Rough River, where Shawnee rocksnails were collected, reached a maximum temperature of 28.6°C in the same year (USGS 2017b). It is important to keep in mind, though, that the temperatures that were lethal to these species (30 and 35°C) both caused much more than 50% mortality; typically, over 50% death in a given time period is considered lethal (Cox and Rutherford 2000).

Information on thermal maxima for freshwater snails is relatively sparse, compared to marine and especially intertidal snails (McMahon 2001). The most comparable studies found activity cessation with chronic exposure to 33.7±3.08°C for Jackson lake springsnails (*Pyrgulopsis robusta* (= *P. idahoensis*), Lysne and Koetsier 2006) and a 96 hr LT50 of 31±0.6°C at constant temperature for New Zealand mud snails (*Potamopyrgus antipodarum*, Cox and Rutherford 2000), both gill-breathing snails like the species in this study. A widely used generalization of gill-breathing freshwater snail thermal tolerance supplies a range of 35 to 40°C as the critical limit (Aldridge 1983).

However, based on the results of the present study as well as the research of Cox and Rutherford (2000) and Lysne and Koetsier (2006), it appears that the lethal temperature range should be lowered, at least for chronic exposure. The lethal temperature for Shawnee rocksnails is definitely below 35°C, and fine-ridged elimia's likely is as well, given that 35°C is actually the LT95. Research like this displays the fact that relying on data from a few species may underestimate the effects climate change will have on freshwater gastropods and how soon those effects will occur as temperatures continue to rise.

While elevated temperatures did not affect righting time for either species (see Figure 3), they did affect the ability of both species to right successfully, with a significant decrease in the proportion of snails able to right themselves at 27°C for Shawnee rocksnails and at 30°C for fine-ridged elimia (see Figure 2). Thus, the snails that were able to right in each species' elevated temperature treatments did not do so any more slowly than those in the control temperature of 20°C, but fewer snails in each group were able to right at all. Increases in failure to right at elevated temperatures have also been documented in sea urchins (Brothers and McClintock 2015), land snails (Gaitán-Espitia et al. 2013), and New Zealand mudsnails (Sharbrough et al. 2017).

While thorough research on the topic is lacking, it has often been assumed that the upside-down position causes gastropods to be more vulnerable to predators and to lose feeding opportunities (Gore 1966; Lemmnitz et al. 1989; Peck et al. 2004). If righting slowly has negative effects on an individual snail, it is conceivable that failing to right altogether would have far more negative effects, with a total loss of the ability to feed or avoid predators. Unless a current happens to flip the snail back over, it will remain in this

vulnerable state. Additionally, our findings suggest that snails with behavioral responses to declining water levels, such as the common river snail (*Viviparus viviparus*; Poznańska et al. 2015), will become stranded on drying sediments during water level fluctuations when temperatures are warm enough to reduce their ability to right themselves. This study assessed only one behavior, but future work should determine whether other snail behaviors, such as velocity or ability to attach to submerged surfaces, are impacted by elevated temperatures as well. Additionally, only adult snails were tested, but it is possible that juvenile snails experience behavioral impacts at lower temperatures than adults, as is true for other invertebrates (Collin and Chan 2016; Wang et al. 2017).

In summer 2016, the average daily temperature in Elkhorn Creek was 28.1°C (USGS 2017a). Based on the results of this experiment, it would only take ten days of temperatures consistently above 30°C to cause 30% of the fine-ridged elimia population in Elkhorn Creek to fail to right each time they were flipped over. We predict that there may be less risk of imminent behavioral effects to the Shawnee rocksnail population in the Rough River as the daily average temperature in the Rough River was only 19.9°C in summer 2016 (USGS 2017b) and Shawnee rocksnails experienced a significant reduction in righting success at 27°C.

The combined implications of increased failure to right and mortality at elevated temperatures are even more concerning in light of the relative inability of snails to migrate to cooler reaches of streams. Snails can only actively move upstream by 0.3 to 1.0 km per year, and it has been suggested that freshwater snails cannot outrun climate warming (Kappes and Haase 2012). For fine-ridged elimia and Shawnee rocksnails, this study provides further evidence of this conclusion because elevated temperatures impair a

behavior important to migration. Snails unable to right themselves when currents or passing animals flip them over will be even more constrained from moving upstream to cooler waters. North American gill-breathing snails already have smaller geographic ranges than lung-breathing snails and less capacity for metabolic compensation of temperature increases (Alexander and Wagoner 2016).

It is necessary to consider the possibility that the increased mortality and failure to right seen in this study may be partly attributable to reduced oxygen concentrations in the elevated temperature treatments (see Table 1). High temperatures are not directly responsible for all negative effects conferred to exposed animals; the associated decline in oxygen saturation contributes as well (Pörtner 2001). Additionally, hypoxia has been found to lower heat tolerance for freshwater snails (Koopman et al. 2016). However, dissolved oxygen is predicted to decrease in freshwaters as climate warming continues (Cox and Whitehead 2009; Fang and Stefan 2009; Bello et al. 2017). Regardless of whether the effects seen in this study were influenced by oxygen concentration in addition to temperature, the implications for freshwater snails are concerning.

With the 74% imperilment rate of North American snail species at last estimate, it is crucial that all elements of the impacts of climate change be understood as best as possible (Johnson et al. 2013). While temperatures are already nearing those that will negatively affect the behavior and survival for Shawnee rocksnails and fine-ridged *elimia*, knowledge of the impending impacts can only aid conservation efforts. The continued collection of data for freshwater snail species is crucial to guide management actions that slow the loss of these interesting and necessary taxa.

CONCLUSION

The findings contained in this dissertation highlight the importance of testing a range of species when determining toxicological and thermal tolerances. Fine-ridged elimia and Shawnee rocksnails only display impaired righting behavior at concentrations of NH_3 that are well above lethal levels for other species, and they recover to normal righting behavior within seven days of returning to ammonia-free water. Several species of freshwater snails would be underprotected by limits based only on the NH_3 tolerance of Shawnee rocksnails and fine-ridged elimia (Goudreau et al. 1993; Alonso and Camargo 2003). Conversely, for the species studied here, the assumption that various gastropods tolerate similar amounts of a toxicant provides unnecessary levels of protection.

Generalizations of gill-breathing snail thermal tolerances (Aldridge 1983) underestimated lethal temperatures for the species tested in this dissertation by up to 5°C . Both populations may suffer increased mortality sooner than would be expected (based on the tolerances of other snail species). Furthermore, this research suggests that they could face frequent failure to right in the near future, as summer temperatures already surpass the temperatures that significantly reduce righting success for Shawnee rocksnails and fine-ridged elimia (27°C and 30°C respectively).

As climate warming continues in the coming years, water levels are predicted to fluctuate to a greater degree (Heino et al. 2009). Near-shore dwelling species like Shawnee rocksnails and fine-ridged elimia may become stranded on dry shorelines when water levels drop while the water is warm enough to impair the snails' righting ability. These impacts are in addition to pre-existing costs of failing to right, such as increased predation risk and lower feeding success (Weldon and Hoffman 1979; Lemmnitz et al. 1989; Orr et al. 2007).

While the snails studied here only displayed behavioral impairments at NH_3 concentrations that are not typically environmentally relevant, future research should continue searching for the underlying cause of these changes to righting behavior, as it may be useful for understanding the risks to more sensitive species. Other taxa, such as crayfish and fish, experience negative impacts to the gills when exposed to NH_3 , even for only 48 hours (Lang et al. 1987; Romano and Zeng 2013). If there was any damage to the gills of Shawnee rocksnails and fine-ridged elimia after 24-hour exposure to NH_3 , it was not apparent in rates of oxygen consumption. Thus, it is more likely that righting behavior is impaired by NH_3 through another mechanism, at least in the first 24 hours.

Based on these findings, freshwater gastropod conservation efforts should be more concerned with climate warming than ammonia pollution. However, it is imperative that the research does not stop here, as other species that have yet to be studied may be even more sensitive to elevated temperatures and NH_3 than this dissertation or previous research would suggest. The most important conclusion to be gained from this dissertation does not pertain to ammonia or water temperature though; the ultimate conclusion is the assertion that to study freshwater snails properly is to study them

thoroughly. Toxicological and climate change studies must continue until sensitivities have been determined for representatives from as many gastropod families and genera as possible.

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APPENDIX A

Table 1. Sample sizes and average righting times (RT) before and after 24-hour exposure to unionized ammonia (NH₃).

fine-ridged elimia (Elimia semicarinata)

NH ₃ -N (mg L ⁻¹)	n (righted ¹)	n (righted ²)	Pre-exposure Avg RT (min)	Post-exposure Avg RT (min)	n (failed to right)
≤0.05	144	143	4.96	4.61	1
0.25	12	12	3.78	3.36	0
0.26	20	20	4.85	4.70	0
0.65	16	15	5.46	3.86	1
0.69	18	17	3.55	4.16	1
0.70	22	22	3.74	3.92	0
1.09	19	18	4.68	2.71	1
1.50	27	26	3.92	3.50	1
1.78	22	22	5.14	6.78	0
1.83	27	24	3.97	4.03	3
2.21	22	22	5.98	5.62	0
3.44	27	27	5.43	3.44	0
3.61	28	28	4.06	3.67	0
4.15	26	26	4.97	5.88	0
5.03	26	25	4.23	4.15	1
6.24	26	26	4.12	5.54	0
9.03	27	25	4.98	7.45	2
12.51	32	30	3.77	5.45	2

n (righted¹) = number of snails that righted themselves within 20 minutes in pre-exposure tests

n (righted²) = number of snails that righted within 60 minutes in post-exposure tests

n (failed to right) = number of snails that failed to right in post-exposure tests

Shawnee rocksnail (*Lithasia obovata*)

NH ₃ -N (mg L ⁻¹)	n (righted ¹)	n (righted ²)	Pre-exposure Avg RT (min)	Post-exposure Avg RT (min)	n (failed to right)
≤0.05	92	75	8.19	10.17	17
0.34	11	8	6.48	7.33	3
0.72	12	12	9.58	5.14	0
0.95	9	7	11.16	10.27	2
1.04	11	8	8.29	10.84	3
1.41	15	10	6.78	10.88	5
1.43	10	9	8.20	12.85	1
1.63	11	9	7.21	8.66	2
1.64	9	9	7.64	13.82	0
1.65	10	8	12.01	8.35	2
2.38	15	13	6.80	17.73	2
3.52	10	5	11.18	15.60	5
3.91	10	7	9.55	14.55	3
4.23	8	6	8.74	15.07	2
4.35	8	7	8.69	18.74	1
6.91	26	21	8.50	6.87	5
8.99	14	10	9.82	9.54	4
12.61	13	5	7.54	15.71	8

n (righted¹) = number of snails that righted themselves within 20 minutes in pre-exposure tests

n (righted²) = number of snails that righted within 60 minutes in post-exposure tests

n (failed to right) = number of snails that failed to right in post-exposure tests

APPENDIX B

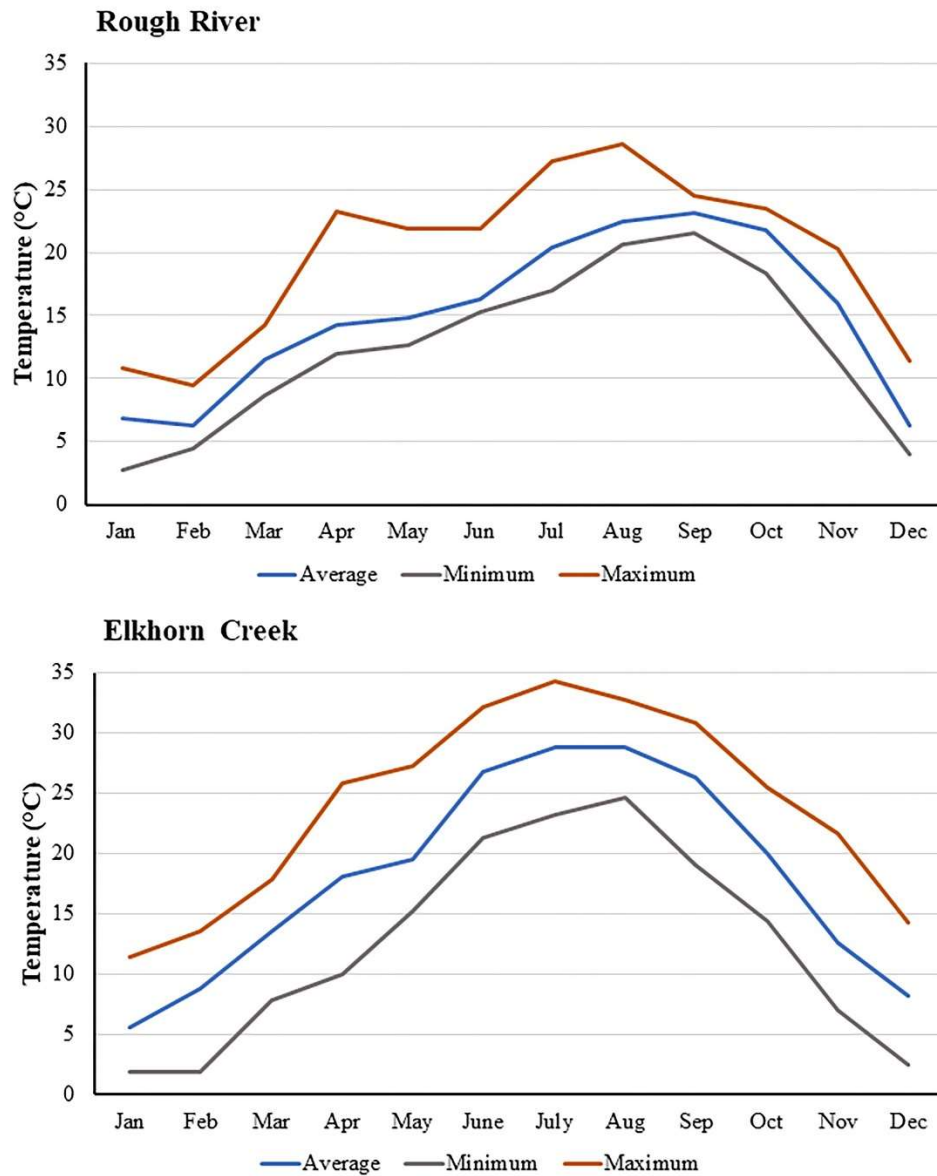


Figure 1. Monthly water temperatures (average, minimum, and maximum) in the Rough River near Falls of Rough, Kentucky, USA and Elkhorn Creek near Frankfort, Kentucky, USA in 2016. All data were obtained from USGS (2017a, 2017b).

APPENDIX C

Chapter II of this dissertation has been published by *Marine and Freshwater Behaviour and Physiology* as follows:

DeWhatley MC, Alexander JE Jr. 2018. Impacts of elevated water temperatures on righting behavior and survival of two freshwater caenogastropod snails. *Mar Freshw Behav Physiol.* 51(4):251–262.

Permission to include this paper in the dissertation was provided on 10/24/2018 by Natalie Davall (natalie.davall@tandf.com.au). The original text of the permission is as follows: “It will be fine to use the article as part of the dissertation. The final PDF or Version of Record cannot be used as this can only be published on the Taylor and Francis website, however, they are free to use the author accepted manuscript (the one accepted after peer review).”

Furthermore, an excerpt from the Author Publishing Agreement (section 4, subsection viii) states that the author retains “the right to include the article in a thesis or dissertation that is not to be published commercially, provided that acknowledgement to prior publication in the Journal is given.”

CURRICULUM VITAE

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Education

Ph.D., Biology Dec 2018
University of Louisville, Louisville, KY
Advisor: James E. Alexander, Ph.D.
Dissertation: Environmental Stressors' Effects on Freshwater Snails

M.S. Non-Thesis, Biology May 2016
University of Louisville, Louisville, KY
Advisor: James E. Alexander, Ph.D.

Bachelor of Science, Biology, *summa cum laude* May 2012
Belmont University, Nashville, TN
Field Biology Emphasis, Minor in Environmental Studies

Teaching Experience

Co-instructor, University of Louisville Jan 2018- May 2018
Introduction to Biological Systems (non-majors)
-design and implement group activities in large lecture (287 students)
-create online pre-lecture activities and post-lecture homework
-design and deliver lectures
-contribute exam questions

Graduate Teaching Assistantship, University of Louisville
Labs Taught:
Laboratory for Introduction to Biological Systems (non-majors) Jan 2017- Dec 2017
Principles of Biology Lab (majors) Jan 2015- Jul 2016
-create and deliver short lectures
-develop assignments to aid learning of concepts and skills
-guide development and analysis of student-planned experiments

Graduate Teaching Assistant Academy
Programs Completed:
1. Introduction to Teaching in Higher Education Sep- Dec 2017
Program for graduate students to develop pedagogical skills and knowledge through weekly interactions with faculty, micro-teaching experiences, and informative lectures and readings

2. Advanced Concepts and Strategies in Post-Secondary Teaching Feb- Apr 2018
Immersive program for graduate students to hone upper level teaching skills and knowledge through bimonthly discussion sessions, independent research of pedagogy literature, and teaching evaluations

Mentor to Undergraduate Research Student May - Aug 2017
Zach Bixler, Junior, University of Louisville
Guide the student in a research program to provide experience in ecological fieldwork, data processing and analysis, and scientific writing

Guest Lecture for Invertebrate Zoology Course Apr 2016
Invited Speaker
University of Louisville, Instructor: Dr. Steve Yanoviak
Lecture topic: Gastropods

Teaching-related Service

Guest Lecture for Undergraduate Biology Majors Nov 2016
Invited Speaker
Belmont University, Nashville, TN
Topic: How to apply to and thrive in graduate school for biology

Research Experience

University of Louisville, Louisville, KY July 2013- Dec 2018
Ph.D. Student; Advisor: James E. Alexander, Ph.D.
Impacts of ammonia and temperature on freshwater snail behavior and physiology.
Skills gained: R (statistics program), respirometry, spectrophotometry

Belmont University, Nashville, TN Jan 2011-May 2012
Undergraduate Research Sequence for Biology Majors
Advisor: Darlene Panvini, Ph.D.
Terrestrial animal diversity in two middle Tennessee freshwater wetlands.

Presentations and Publications

DeWhatley, MC and JE Alexander, Jr. 2018. Impacts of elevated water temperatures on righting behavior and survival of two freshwater caenogastropod snails. *Marine and Freshwater Behaviour and Physiology*. 51(4):251–262.

DeVries (DeWhatley), MC and JE Alexander, Jr. 2017. Impacts of unionized ammonia on righting behavior in two species of freshwater snails. Oral presentation delivered at the 37th Annual Midwest Ecology and Evolution Conference, Champaign, IL.

DeVries, MC and JE Alexander, Jr. 2016. Effects of unionized ammonia on righting time in *Elimia semicarinata*. Oral presentation delivered at the 10th Annual Kentucky Freshwater Mollusc Meeting, Frankfort, KY.

DeVries, MC. 2016. Sublethal impacts of nonionized ammonia on the freshwater snail *Pleurocera acuta*. Three minute oral presentation delivered at the Graduate Student Regional Research Conference, Louisville, KY.

DeVries, MC and JE Alexander, Jr. 2015. Impacts of nonionized ammonia on righting time in the freshwater snail, *Pleurocera acuta* (Pleuroceridae). Poster presentation delivered at the 101st Annual Meeting of the Kentucky Academy of Science, Highland Heights, KY.

DeVries, MC. 2014. Effects of nonionized ammonia on movement behavior of a freshwater nonpulmonate snail: A research proposal. Oral presentation at the 9th Annual Kentucky Freshwater Mollusc Meeting, Mammoth Cave, KY.

DeVries, MC and D Panvini. 2012. Terrestrial animal diversity in two middle Tennessee freshwater wetlands. Oral presentation at the Tennessee Academy of Science Middle Collegiate Division Meeting for Undergraduate Research, Nashville, TN.
1st Place in Ecology/ Environmental Category

Research Grants

BGSA Research Grant (\$100) 2017
Biology Graduate Student Association, University of Louisville
Proposal title: "Impacts of unionized ammonia on oxygen consumption in two species of freshwater snails"

Graduate Research Fund (\$300) 2015
Graduate Student Council, University of Louisville
Proposal title: "Effects of nonionized ammonia on righting time of the freshwater snail, *Pleurocera acuta*"

Large River Fund (\$500) 2015
University of Louisville
Proposal title: "Effects of nonionized ammonia on the righting behavior of a locally declining freshwater snail"

Marcia Athey Fund (\$312) 2015
Kentucky Academy of Science
Proposal title: "Effects of nonionized ammonia on the behavior of a locally declining freshwater snail"

Awards and Scholarships

Doctoral Dissertation Completion Award 2018
Supports outstanding Ph.D. candidates in the final semester.
Competitive award, nomination by Ph.D. candidate's department.

Service Award 2017
Awarded to a member of the Biology Graduate Student Association at University of Louisville for service to the department and/or university. Voted by members of the association.

University of Louisville Graduate Fellowship 2013
Competitive award, nomination by Ph.D. student's department.

Professional Service

Biology Graduate Student Association
University of Louisville

Member	Sep 2013- Dec 2018
Treasurer / Vice President	Apr 2017-Apr 2018
President	Sep 2016- Apr 2017
Fundraising Chair	Apr 2016- Apr 2017
Secretary	Apr 2015- Apr 2016

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