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Water Acidification and Pathogen Exposure Negatively Impact Innate Immunity in American Bullfrog (*Lithobates catesbeianus*) Tadpoles

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ABSTRACT

Freshwater acidification, an issue largely linked to industrialization and human activity, threatens freshwater environments and the organisms that inhabit them. The combination of acidification and pathogens already present in freshwater may increase the threat by altering immune defense in these organisms. We exposed American bullfrog (*Lithobates catesbeianus*) tadpoles to acidified water (pH 5.5), and 1.8 mL of a solution containing 100 colony forming units per 300 μL of *Aeromonas hydrophila*, a common water bacterium and amphibian pathogen. We monitored development, growth, and one parameter of immune defense, as measured by bacterial killing assays performed on whole blood samples from tadpoles among different treatment groups. Exposure to each acidic conditions and *A. hydrophila* significantly decreased *A. hydrophila* killing ability in *L. catesbeianus*. Our data suggest that *L. catesbeianus* is susceptible to decreased immune defense in the presence of stressors, both natural and those that result from anthropogenic activity.

INTRODUCTION

Freshwater acidification is an issue that has been gaining attention for decades because it poses a threat to freshwater ecosystems and associated organisms. Freshwater acidification is a process that occurs when rain mixes with nitrogen oxides and sulfur dioxides in the atmosphere, forming acid rain. The acidic compounds fall onto soil and into water, lowering the pH of both and resulting in freshwater and soil acidification (Moiseenko and Gashkina 2011). Although acid rain occurs naturally to some extent, it has become increasingly prevalent with the rise in anthropogenic activity during the past century (Schindler 1988; Moiseenko and Gashkina 2011). Common anthropogenic practices include the application of nitrogen-containing fertilizers to crops, which releases chemicals into soil, and fossil fuel combustion, which emits sulfur dioxide, carbon dioxide and nitrogen oxide into the atmosphere as by-products. Runoff from fertilizer-sprayed fields enters freshwater, lowering the overall pH and acidifying the water (Vitousek et al. 1997). During fossil fuel combustion, sulfur dioxide and nitrogen oxide enter lakes and streams in the form of acid rain formed from industrial emissions (Schindler 1988; Moiseenko and Gashkina 2011).

Amphibians are particularly sensitive to changes in freshwater chemistry as a result of their semi-permeable skin, and are consequently used as model organisms in many environmental studies (Räsänen et al. 2002; Miaud et al 2011; Katzenberger et al. 2014). The effect of lowered pH has been explored at various biological scales in several amphibian species. For example, Räsänen et al. (2002) found that acidic conditions negatively affected growth and survival in European common frog (*Rana temporaria*) tadpoles. Additionally, Vatnick et al. (2006) found that lowered water pH resulted in decreased immune defense in adult northern leopard frogs, *Lithobates pipiens*, specifically noting a reduction in phagocytosis by leukocytes. Krynak et al. (2015) found that acidification significantly changed microbial communities on larval American bullfrog (*Lithobates catesbeianus*) skin, which may impact the tadpoles' ability to resist disease. These findings suggest that a decrease in pH may act as a physiological stressor in amphibians, resulting in reduced growth and slowed development, as well as altered immune defense (Räsänen et al. 2002; Vatnick et al. 2006; Krynak et al. 2015).

In a freshwater environment, amphibians come into contact with many potential pathogens. Typically, these pathogens do not cause infection in the animals. However, if the amphibians are stressed, opportunistic bacteria may be able to act on the weakened immune system and cause infection (Mauel et al. 2002). *Aeromonas hydrophila* is among the most prolific amphibian and fish pathogens (Rivas, 2016). As an opportunistic pathogen, individuals with impaired or compromised immune systems are most susceptible (Mauel et al. 2002; Esch and Hazen 1980; Altwegg and Geiss 1989). *A. hydrophila* is able to grow and survive in water with a pH range from 5 to 9, and is able to survive in most environments (Hazen et al. 1978). *A. hydrophila* infection in frogs results in the development of a disease known as red leg syndrome or red leg disease. Red leg syndrome in adults presents as a red discoloration of the tissues of the hindlegs, and in tadpoles it presents as red discoloration in the abdomen and base of the tail (Cunningham et al., 1996). This red coloration is a result of hemorrhaging within the capillary beds of these tissues. If the disease progresses unchecked, and without recovery, the afflicted organism becomes septic and eventually dies as a result of hemorrhagic septicemia (Cunningham et al., 1996). Gabor et al. (2013) found that elevated glucocorticoid levels in two species of tadpoles were positively correlated with infection by the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* (Bd). Venesky et al. (2012) showed that stress in the form of a low

protein diet in southern leopard frog tadpoles (*Lithobates sphenocephalus*) led to suppressed immune function, manifesting as a lessened inflammatory response and decreased bacterial killing ability against Bd. Based on these studies, it can be concluded that physiological stressors can suppress immune function and therefore likely contribute to disease (Carey et al. 1999).

Though the aforementioned studies contribute to the scientific knowledge of the relationship between stressors and physiology in freshwater organisms, many of them focus on only one stressor at a time. In their natural settings, tadpoles are exposed to multiple stressors, including, but not limited to, water acidification, pathogen exposure, predation, and fluctuating water levels. While our study did not address all of these stressors, it did introduce two stressors that are present in many freshwater systems and have been previously documented to have significant negative impacts on amphibian immune defense. Moore and Klerks (1998) studied the interactive effect of high temperature and low pH on the physiology of *Acris gryllus* and *Lithobates clamitans* tadpoles, but to our knowledge, the effect of water acidification and pathogen exposure on tadpole physiology has not previously been studied.

For our study, we used acid and pathogen exposure as our two stressors, based on the findings of Vatnick et al. (2006), Venesky et al. (2012) and Gabor et al. (2013). We predicted that American bullfrog (*Lithobates catesbeianus*) tadpoles exposed to water with a pH of 5.5 would exhibit decreased immune defense against *A. hydrophila* compared to those unexposed to acid. We also hypothesized that the tadpoles facing multiple stressors (acid and *A. hydrophila*) would have even further decreased immune defense compared to those facing no stressors or only one stressor (Davis et al. 2008).

METHODS

***Lithobates catesbeianus* tadpole husbandry.** The American bullfrog (*Lithobates catesbeianus*) tadpoles that we used for our experiment were raised in a pond aquaculture facility in Ohio (Pond Megastore, Canton, OH) and shipped to Decatur, Illinois on 19 April 2016 for our experiment at Millikin University. Upon arrival, they were placed into a 189.3 L (50 gal) tank containing approximately 63 L of tap water aged 3 days. The tadpoles (n = 150) were housed in this tank overnight before experimental setup began. On 20 April 2016, we separated 20 rectangular plastic containers (2129.29 mL / 72 oz) into groups of five to set up each of our four treatment groups (group 1: control, group 2: pathogen, group 3: acid, group 4: acid and pathogen; Table 1). Each container was filled with 1800 mL of tap water aged 3 days (pH 7.3, ± 0.3, ~23˚C), and three tadpoles were transferred to each container using a small fish net. Gosner stages ranged from 31-40 (mean = 37) and snout-to-vent length (SVL) ranged from 3.55-4.85 cm (mean = 4.38 cm) within the entire sample pool, but similar-staged and similar-sized tadpoles were grouped together within containers. We fed the tadpoles ad libitum with high-protein algae discs (2 cm in diameter, approximately 1.5 discs per 3 tadpoles, given every other day; Aquatic Foods, Inc.) upon initial setup, and after each water change for the entirety of the experiment. The guaranteed analysis of this food was minimum 30% crude protein, minimum 7.5% crude fat, maximum 4.0% crude fiber, and maximum 10.0% moisture.

After the initial partitioning of tadpoles into containers, we allowed five days for acclimation and mucosomal buildup on containers, with full water changes every three days. To perform water changes, we transferred the tadpoles with a fish net to a separate container holding enough aged water to fully immerse them. We then disposed of the old water and uneaten food, and refilled the original container with 1800 mL of aged tap water. The tadpoles were transferred back into their original containers and given more algae discs. Following the initial five days of acclimation, we performed full water changes every other day. After beginning treatments, we used separate nets and temporary containers for pathogen and non-pathogen tadpoles to avoid cross-contamination. We left the containers unlidded with a sheet of mesh screen placed over the top of all containers for the entirety of the experiment. None of the tadpoles died before we performed the bacterial killing assay.

Acidification of water. On 25 April 2016, we administered the acid treatment to treatment groups 3 and 4 (Table 1). In order to lower the pH of the water for these two groups to a pH of approximately 5.5, we measured 600 mL of aged tap water in a separate container and, using a dropper bottle, added one drop of 10% sulfuric acid (H_2SO_4) per 600 mL of aged tap water and mixed. We tested the pH of the acidified water using an HI 8014 pH meter to confirm that the pH was around 5.5 (± 0.3). We added 1800 mL of the acidified water to each of the containers in treatment groups 3 and 4 using the described method of acidified water preparation every time we performed water changes for the duration of the experiment. Aged tap water from this lab (non-acidified) has a pH of 7.3 (± 0.3), with an average temperature of 22.8°C, salinity of 0.3ppt, and dissolved oxygen of 9.20 mg l^{-1}.

Introduction of pathogen, *Aeromonas hydrophila*. On 25 April 2016, we added aliquots of the pathogens to treat-

Table 1. The administration of treatments (control, acid, pathogen, acid + pathogen) to American bullfrog tadpoles, *Lithobates catesbeiana*.

Tadpole treatment group number	Acid treatment (1 drop 10% H_2SO_4 / 600 mL)	Pathogen treatment (100 CFU *Aeromonas hydrophila* / 300 μL)
1 (control)	-	-
2	-	+
3	+	-
4	+	+

ment groups 2 and 4 (Table 1). To do this, we diluted a stock solution (300 colony-forming units/300 mL) of *Aeromonas hydrophila* to a concentration of 100 colony-forming units (CFU) per 300 μL. The *A. hydrophila* strain used in this study was ATCC® 49140™ (Microbiologics, Inc.). After performing the appropriate water changes (regular aged tap water for group 2 and acidified aged tap water for group 4) and feeding the tadpoles in each group, we added 1800 μL of the diluted bacteria solution to each of the containers in groups 2 and 4, for a final concentration of 3.3×10^{-4} CFU/μL (0.33 CFU/mL) in each container. Diluted bacteria solution was added to these containers on a weekly basis. Though the environmental concentration of *A. hydrophila* ranges greatly depending on water parameters (e.g., salinity, turbidity, concentration of organic matter, etc.), the final concentration used in our study was located within reported naturally occurring ranges (Fliermans et al. 1977; Hazen et al. 1978; Kaper et al. 1981).

Bacterial killing assay. On 6 May 2016, we collected blood from tadpoles for use in our bacterial killing assay. We prepared an anesthetic bath using 1.8 g of Tricaine-S (MS-222) mixed with 900 mL of water. We transferred each tadpole to the anesthetic bath using a small fish net. Once each tadpole was unconscious, we recorded the Gosner stage (Gosner 1960) and used a ruler to measure the SVL in cm. Using a hypodermic needle, we performed cardiac puncture and collected blood from the tadpoles using a microhematocrit capillary tube. We transferred the blood to snap-top microcentrifuge tubes using a Hamilton™ syringe. Each tadpole was then euthanized via anesthetic (MS-222) overdose and subsequently transferred to a lidded container with 70% EtOH. The blood samples were vortexed, placed in a microcentrifuge tube rack, and frozen until needed for the bacterial killing assay. To complete the bacterial killing assay (procedures adapted from Liebl and Martin 2009), we added 100 μL of Phosphate-buffered saline (PBS) in each well of a polystyrene 96-well plate. We then vortexed the blood sample tubes and transferred 10 μL of each blood sample to duplicate wells on the plate (20 μL of each sample total), and added 10 μL of the stock *A. hydrophila* solution (non-diluted, 300 CFU/300 mL) to each well. We added 110 μL of PBS in separate duplicate wells (220 μL in total) and added 10 μL of the stock *A. hydrophila* solution to act as a positive control for the assay, allowing us to determine background optical density. We also used wells with only broth to determine potential contamination and as a negative control. Then, we incubated the plates at 27˚C for 30 minutes. After incubation, we used a Finnpipette™ multichannel pipette to transfer 100 μL of tryptic soy broth to each row of wells on the plate. After filling each well, we read the plates on a BioRad iMark microplate spectrophotometric reader at 415 nm and gathered data. The plates were then covered with Parafilm® and incubated at 37˚C in a Precision™ incubator for 24 hours. After the 24-hour incubation period, the plates were reread at 415 nm. Bacterial killing ability (BKA) was calculated as the change in optical density from initial reading to the 24-hour reading from the control wells with only bacteria and broth minus the average change in optical density from initial reading to the 24-hour reading for the duplicate samples, divided by the difference in optical density from the controls. These values were multiplied by 100 to generate a percentage of bacteria killed (Liebl and Martin 2009). There was no growth in the negative wells.

Statistics. We used general linear mixed models with *A. hydrophila* killing ability as the dependent variable; pH group and *A. hydrophila* exposure as fixed factors; and Gosner stage, snout-to-vent length (SVL), and length of time each blood sample was frozen as covariates to determine whether *A. hydrophila* killing ability differed among treatments, when considering varying Gosner stages, SVL and sample freeze times for the tadpoles. The container number was included as the random variable. We used Tukey's post hoc analysis to compare the differences in mean BKA among treatment groups. All statistical analyses were completed using SPSS. We considered a p-value of less than 0.05 to be statistically significant.

RESULTS

We found a significant effect of treatment on A. hydrophila killing ability ($F_{3,54} = 4.55$, $P = 0.002$; Fig. 1). Tukey's post hoc analysis revealed that the control tadpoles had significantly higher *A. hydrophila* killing ability (25.6% ± 4.3%) than each of the treatments (pathogen:

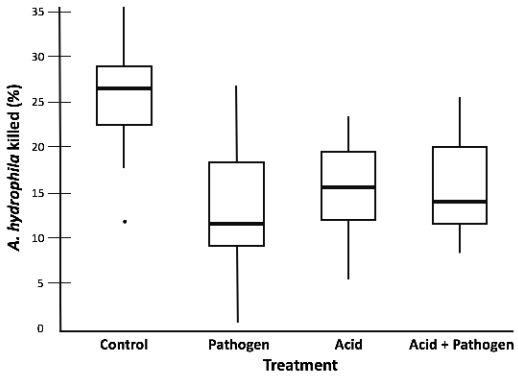

Figure 1. The effect of exposure to *Aeromonas hydrophila*, acidified water, or a combination of those stressors, on *A. hydrophila* killing ability (BKA) in American bullfrog tadpoles, *Lithobates catesbeianus*. Data are presented as box and whisker plots, where boxes encompass values between the 25th percentile and 75th percentile, horizontal lines represent median values, and whiskers represent the range of values. Control tadpoles had a significantly greater BKA than pathogen, acid, and acid + pathogen tadpoles ($P < 0.05$ in each case).

11.44% ± 3.3%; $P = 0.024$, acid: 14.08% ± 4.0%; $P = 0.042$, acid and pathogen: 13.5% ± 4.2%; $P = 0.039$). However, there were no significant differences in *A. hydrophila* killing ability among the non-control treatments ($P > 0.42$ in each case).

DISCUSSION

Our hypotheses at the beginning of this experiment were that water acidification would have a negative impact on the immune defense of *Lithobates catesbeianus* tadpoles, and that multiple stressors (acid and *Aeromonas hydrophila*) would cause further decreased immune defense. Räsänen et al. (2002) found that *Rana temporaria* tadpole growth and survival were negatively impacted when decreases in water pH were observed. This is likely due to the role of acidity as a physiological stressor in amphibians. Likewise, Vatnick et al. (2006) found that acidity acted as an immune disruptor in northern leopard frogs, specifically that phagocytosis of leukocytes was reduced. Because we used whole blood in our bacterial killing assay, reduced phagocytosis could explain the effects of increased acidity we observed in the study. The stress response of vertebrates is mediated by glucocorticoids. When a stressor is perceived, glucocorticoids are released, which can have a negative impact on circulating lymphocyte numbers if the stressor is long-term (Davis et al. 2008; Dhabhar 2008). While it is generally accepted that acute stress augments immune defenses, several studies have found that acute stress can also decrease circulating lymphocyte numbers (Kanemi et al. 2005; Dhabhar 2008). Because lymphocytes are an essential component of innate and acquired immunity, a decrease in lymphocytes can result in suppressed immune function and impaired ability to fight off infection. Thus, chronic stress, and perhaps also acute stress, can suppress immune function (Kanemi et al. 2005; Davis et al. 2008). Because physiological stressors can alter immune defense in organisms, stress is linked to disease.

Acid acts as both a physiological stressor and an immune disruptor; therefore, we hypothesized that acid would decrease immune defense, as measured by *A. hydrophila* killing ability, and that the added presence of a pathogen stressor in acidic conditions would decrease the immune defense of the tadpoles even more than acid alone. The hypothesis that multiple stressors would further decrease immune defense was not supported in this study; we found no significant differences in *A. hydrophila* killing ability among non-control (low pH, *A. hydrophila* exposure, combination low pH and *A. hydrophila* exposure) treatments. Our results suggest that the presence of stressors causes decreased immune defense in *L. catesbeianus*, but the nature of the stressor is not as important.

It is important to note that this study involved a large amount of handling of the tadpoles. Physical disturbance by handling is known to cause stress in vertebrate study organisms (Pickering et al. 1982; Barton et al. 1987; Barton and Iwama 1991; Dobrakova and Kvetnansky 1993). For example, Barton et al. (1987) found that juvenile rainbow trout (*Salmo gairdneri*) exposed to acute daily stress by handling exhibited increases in plasma cortisol and glucose, and long-term decreases in circulating lymphocytes. Likewise, Pickering et al. (1982) found that acute handling of brown trout (*Salmo trutta*) resulted in decreased circulating lymphocytes, along with an increase in plasma cortisol and glucose. Dobrakova and Kvetnansky (1993) found that daily handling of rats also induced a stress response, measured by an increase in corticosteroids. Because our study involved a large amount of tadpole handling, it is possible that the decrease in bacterial killing ability observed in our tadpoles was partially due to handling stress. However, even the control tadpoles were introduced to this same handling stressor, so any effects of handling stress should have also been reflected in the control tadpoles, shifting the baseline for our tadpoles' bacterial killing ability. There is, however, the possibility that the tadpoles in our study became acclimated to the handling stress, and the introduction of a novel stressor (acid alone, pathogen alone, or acid and pathogen combined) induced a facilitation response in the non-control tadpoles, invoking a more severe stress response and therefore further decreasing bacterial killing ability (Dobrakova and Kvetnansky 1993). This would have appeared as a decreased bacterial killing ability in all but the control tadpoles, which is what we observed. Thus, it is possible that the handling of the tadpoles contributed to the decreased bacterial killing ability among the tadpoles exposed to one or more stressors (acid and pathogen). However, in a similar study with Cuban tree frog tadpoles (*Osteopilus septentrionalis*), a comparably large amount of handling was involved, and there were no significant effects on bacterial killing ability observed in any of the tadpoles (control, acid, pathogen, combination acid/pathogen; Peck and Wilcoxen, 2018). In order to determine if facilitation responses to handling stress occurred in our study, further research addressing handling stress in tadpoles would need to be conducted.

Another issue that should be addressed is that of pathogen exposure in our study. While we added *Aeromonas hydrophila* to the containers of tadpoles in groups 2 and 4, the addition of the pathogen to this water most likely increased the concentration of *A. hydrophila* present in the water rather than introducing a novel pathogen. Hazen et al. (1978) sampled water from 147 sites across 30 states in the United States, and found that *A. hydrophila* was present in all but twelve sampling locations. Seven of these twelve sites were extreme environments – either hyperthermic, hypersaline, or extremely polluted (Hazen et al. 1978). It is suggested, therefore, that *A. hydrophila* has a role in the natural functioning of aquatic environments, based on its presence and abundance across many different aquatic environments and systems. Additionally, *A. hydrophila* is commonly found in low concentrations in drinking water, which was the source of our aged water for each treatment group (van der Kooij, 1988). Based on these findings, it is likely that *A. hydrophila* was present in the water

of all of our tadpoles, but in greater concentrations in the treatment groups to which additional *A. hydrophila* was added. Since it is likely that our tadpoles had already been exposed to environmental levels of *A. hydrophila* prior to our experiment, an investigation into the effects of *A. hydrophila* exposure on acquired immunity is one possible area of future research. Most studies of the effects of stressors on larval amphibians also involve a close examination of growth and development. Because our study was carried out over two weeks with American bullfrog tadpoles, with a larval period of two to three years (Collins 1979), neither body size nor developmental stage changed for tadpoles in this study. However, further investigation, over a period of time that would allow examination of the impacts of these stressors (acid and *A. hydrophila*) on growth and development of this species would also provide better insight into potential impacts beyond innate immunity.

While our tadpoles experienced decreased bacterial killing ability when faced with increased *A. hydrophila* exposure, they were also similarly negatively affected by acidified water (Fig. 1). Central Illinois is an area heavy in limestone and dolomite, and is therefore somewhat buffered from the effects of acid precipitation due to the calcium carbonate present in its waterways (Kempton et al. 1991; McConkey Broeren et al. 1991). However, in areas of the United States where limestone does not naturally occur (including part of the native range of *L. catesbeianus*), freshwater acidification still poses a threat. Additionally, a recent study by Weiss et al. (2018) suggests that, similar to marine waters during the process of ocean acidification, bodies of freshwater have also begun experiences decreases in pH as a result of absorbing atmospheric carbon dioxide. Typically, freshwater acidification is linked to nitrogen and sulfur-based compounds. As a result, in recent decades, efforts have been made to reduce anthropogenic nitrogen and sulfur emissions (Schindler 1988; Moiseenko and Gashkina 2011). However, freshwater acidification is still an issue today. With the evidence presented by Weiss et al. (2018) for carbon emission-based freshwater acidification, it is possible that the issue will continue to pose a threat to freshwater ecosystems and organisms such as Lithobates catesbeianus, a species that appears to be susceptible to stressors, for decades to come. While more research is needed to definitively interpret the findings of our study, it is clear that *L. catesbeianus* is negatively affected by stressors in its environment.

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