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Effects of water acidification and pathogen exposure on innate immunity in Cuban tree frog (Osteopilus septentrionalis) 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 additional stressors already present in freshwater may increase the threat. We exposed Cuban tree frog (Osteopilus septentrionalis) tadpoles to acidified water and Aeromonas hydrophila, a common water bacterium and amphibian pathogen. We monitored development, growth, and immune defense among tadpoles from different treatments, and performed bacterial killing assays on whole blood samples from these tadpoles. We found that neither acidity nor pathogen exposure, nor a combination of the two, had a statistically significant effect on immune defense in O. septentrionalis, as measured by A. hydrophila killing ability, when considering varying Gosner developmental stages and snout-to-vent lengths. There was a trend toward reduced bacterial killing ability in animals receiving any of the stressors compared to those in the control group. Our results could hold implications for the future of native species as a result of increased tolerance to environmental stressors in this invasive species.

Keywords: *innate immunity; amphibian; acid rain; toxicology.*

What is already known

Freshwater acidification is increasing with the increased burning of fossil fuels. Acidification can reduce growth and survival in amphibians. Acidification can also have sub-lethal effects on amphibians by altering inflammatory responses and skin microbial communities.

What this study adds

Acidification did not significantly alter growth or development in an invasive tree frog species. There is a trend toward reduced innate immune defense against a known amphibian pathogen in animals raised in acidic conditions.

INTRODUCTION

Freshwater acidification is an issue that has been gaining attention for decades because it poses a threat to freshwater ecosystems and the organisms that form them. Freshwater acidification is a process that occurs when rainwater mixes with naturally existing nitrogen oxides and sulfur dioxides in the atmosphere. These compounds dissolve in rainwater or other types of precipitation to form acidic solutions known as 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 regardless of human activity, due to naturally occurring gases in the atmosphere, 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 use of herbicides and pesticides in farming (which releases chemicals into soil) and fossil fuel combustion (which emits sulfur dioxide, carbon dioxide and nitrogen oxide into the atmosphere as by-products). The chemicals used to reduce weeds and insects in crops enter freshwater bodies by way of runoff from fields, while sulfur dioxide and nitrogen oxide enter lakes and streams in the form of acid rain formed from industrial emissions. Another contributing factor to freshwater acidification is the application of nitrogen-containing fertilizers to crops. As with herbicides and pesticides, runoff from fertilizer-sprayed fields enters

freshwater, lowering the overall pH and acidifying the water (Vitousek et al. 1997). All methods of acidification are concerning due to the threat they pose to aquatic wildlife; the effects of acidification (either directly or by runoff) can be detrimental to aquatic organisms.

Amphibians are particularly sensitive to changes in freshwater chemistry as a result of their permeable skin, and are consequently used as model organisms in many environmental studies (Räsänen et al. 2002; Miaud et al. 2011). The effect of lowered pH has been explored at multiple levels in several amphibian species. For example, Räsänen et al. (2002) found that acidic conditions negatively affect growth and survival in European common frog (*Rana temporaria*) tadpoles. Additionally, Vatnick et al. (2006) found that lowered water pH results in decreased immune defense in adult Northern leopard frogs, *Rana pipiens*, as evidenced by a less effective inflammatory response when injured, and a decreased amount of circulating white blood cells. Krynak et al. (2015) found that acidification significantly changes microbial communities on larval American bullfrog (*Rana catesbeiana*) skin, which may impact the tadpoles' ability to resist disease. These findings suggest that, while a decrease in water pH can be damaging to amphibians, it is not necessarily lethal. Independent of lethality, a decrease in pH can act as a physiological stressor in amphibians, resulting in reduced growth and slowed development, as well as altered immune defense (Vatnick et al. 2006; Krynak et al. 2015). The negative effect of physiological stressors on immune defense is a central topic in ecological research and has been explored in depth.

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). Because lymphocytes are an essential component of acquired immunity, a decrease in lymphocytes can result in suppressed immune function and impaired ability to fight off infection. Thus, chronic stress can suppress immune function (Davis et al. 2008). Because physiological stressors can alter immune defense in organisms, stress is linked to disease. Gabor et al. (2013) found that elevated glucocorticoid levels in two species of tadpoles are correlated with infection by the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* (*Bd*). Similarly, a study done by Venesky et al. (2012) showed that a low protein diet in southern leopard frog tadpoles leads to suppressed immune function, manifesting as a lessened inflammatory response and a decreased ability of the blood to fight off *Bd*. Though the suppressed immune function may be a direct result of protein deficiency (causing an inability to mount a proper immune response), it is likely that the stress of poor nutrition also contributes to the suppressed immune function in southern leopard frog tadpoles (Venesky et al. 2012). Mauel et al. (2002) also reported on the effects of stress in amphibians on immune function and disease transmission. 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 and face immune suppression as a result of the stress, opportunistic bacteria may be able to act on the weakened immune system and cause infection (Mauel et al. 2002). Based on these studies, it can be concluded that physiological stressors can suppress immune function and therefore likely contribute to disease.

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. While this is helpful in determining the main effects of a stressor on organism physiology, in order to fully assess the physiological condition of an organism with regards to its environment, one must address possible interactions between different stressors. Venesky et al. (2011) studied growth, development and disease transmission in two species of tadpoles exposed to *Bd*, but pathogen exposure was the only stressor studied. 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 have been previously documented to have significant impacts on amphibians, and two that are ever-present in many freshwater systems. Moore and Klerks (1998) studied the interactive effect of high temperature and low pH on the physiology of *Acris gryllus* and *Rana clamitans* tadpoles, but to our knowledge, the effect of water acidification and pathogen exposure on tadpole physiology has not previously been studied. Another aspect of this

study that is different from existing literature is that we measured immune defense in response to multiple stressors by looking at the innate immune response to a known amphibian pathogen. Vatnick et al. (2006) studied the immune response of adult *R. pipiens* in the presence of water acidified using a buffer solution, but this study did not address the effects of acid on immune response in the presence of a true amphibian pathogen. Additionally, the measure of immune defense in many of the other studies is inflammation in response to injection with thioglycollate, or some other foreign antigen, which are substances that do not occur naturally in freshwater environments and therefore would likely not be naturally encountered by amphibians. We aimed to study the effects of multiple stressors on immune defense in tadpoles by measuring tadpole bacterial killing ability of a known amphibian pathogen, while also taking into account varying developmental stages and differing growth among the tadpoles.

For our study, we used acid as the main stressor, as the main topic of study was water acidification. Pathogen exposure was the other stressor that we used, based on the findings of Venesky et al. (2011) and Gabor et al. (2013). The pathogen that we used was *Aeromonas hydrophila*. *A. hydrophila* is a species of water bacteria that is found in most bodies of freshwater. It is able to grow and survive in water with a pH range from 5 to 9. It is a hardy species, and it can survive in many different aquatic environments (Hazen et al. 1978). *A. hydrophila* has also been recognized as an amphibian pathogen, causing a condition known as red-legged disease, but it is an opportunistic bacterium, meaning that infection is likely to occur only if the host has already been affected by stress or disease (Mauel et al., 2002). Vatnick et al. (2006) came to the conclusion that environmental stressors (acid in that particular study) may lead to decreased host condition and provide the chance for opportunistic pathogens to infect the host. Based on these studies, *A. hydrophila* was the ideal pathogen to use for this study. We hypothesized that, in Cuban tree frog (*Osteopilus septentrionalis*) tadpoles, the tadpoles exposed to acid would exhibit decreased immune defense against *A. hydrophila* compared to those unexposed to acid. We also hypothesized that the tadpoles facing multiple stressors would have even further decreased immune defense compared to those facing no stressors or only one stressor, based on the fact that stress causes decreased immune defense (Davis et al. 2008). We thought that increased stressors would cause greater stress in the tadpoles and result in greater immunosuppression. We also hypothesized that exposure to acid in *O. septentrionalis* would cause decreased growth, based on the findings by Räsänen et al. (2002).

METHODS

Osteopilus septentrionalis husbandry

On May 12, 2015, Cuban tree frog (*Osteopilus septentrionalis*) egg masses were collected by a permitted collector from natural bodies of freshwater in Naples, Florida, USA. When the eggs hatched, the tadpoles were separated into groups of five and transferred into containers holding aged tap water. To transfer the tadpoles from the original container to the new ones, a mesh screen was placed under them and lifted, minimizing damage to their bodies and avoiding direct contact between the experimenter's skin and the tadpole skin. The tadpoles were housed in the containers and given partial water changes every three days until the experimental setup began. For the entirety of the study, they were fed high protein algae discs, *ad libitum*, in order to prevent decreased immune response as a result of low protein diet (Venesky et al. 2012). On May 26, 2015, we separated 40 Pactiv® BPA-free 473.18 ml (16 oz) round containers into groups of ten to set up each of our four different treatment groups (group 1: control, group 2: pathogen, group 3: acid, group 4: acid and pathogen). Each container was filled with 240 ml of tap water aged three days and 60 ml of water from the tadpoles' original housing container, which established a healthy microbial community in each container. We then selected 120 tadpoles of approximately equal size, all between Gosner developmental stages 25-27, the onset of hind limb bud development in larval anurans (Gosner 1960). We transferred three tadpoles of equal size into each container, using the same mesh screen method of transfer as before. The tadpoles were given a week to acclimate to the new containers before treatments began, with 50% water changes and feeding occurring every other day. By only changing half of the water during this time, the tadpoles were able to develop a natural mucosome on the walls of their containers. Any food that was not consumed between water changes was removed using mesh screen.

After the initial week of acclimation, we conducted full water changes every other day

for each of the containers, using 300 mL aged tap water per container. To conduct the full water changes, we used mesh screen to transfer the tadpoles to a temporary container holding enough aged water to fully immerse the tadpoles. We emptied the old water from the permanent container, and used a beaker to add 300 ml of fresh aged water to the container. We then poured the water and tadpoles from the temporary container into the freshly changed water. After beginning the treatments, we used separate temporary containers, beakers and mesh screens for each of the treatment groups to avoid cross-contamination among tadpoles from differing treatment groups. The containers were left unlidded, but covered by a sheet of mesh screen for the entirety of the experiment until the tadpoles reached at least Gosner stage 41, the point at which forelimbs become visible but have not yet emerged (Gosner 1960). Fifteen of the original 120 *O. septentrionalis* tadpoles died during the three months of experimentation before the bacterial killing assay, leaving an overall survival rate of 87.5%.

Acidification of water

On June 4, 2015, we administered the acid to treatment groups 3 and 4. 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. We added 300 ml (*O. septentrionalis*) 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. The pH of the non-acidified, aged tap water was approximately 7.3.

Introduction of the pathogen, Aeromonas hydrophila

On June 15, 2015, we added aliquots of the pathogens to treatment groups 2 and 4. To do this, we diluted a stock solution of *Aeromonas hydrophila* to a concentration of 100 colony-forming units per 300 µL. 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 used a P1000 micropipette to add 300 µl of the diluted bacteria solution to each of the containers in groups 2 and 4. Diluted bacteria solution was added to these containers on a weekly basis.

Aeromonas hydrophila bacterial killing assay

On August 5, 2015, the first tadpole of the surviving 105 reached Gosner stage 42, at which time the forelimbs emerge. We moved this froglet to a separate container with a wet paper towel in the bottom, and placed a lid with air holes over the top of the container. Between August 5 and August 31, 2015, we repeated this procedure for all tadpoles that reached stage 42 or higher. On August 10, 2015, we began the preparation for our bacterial killing assays. We first prepared an anesthetic bath for the tadpoles by adding Tricaine-S (Tricaine Methanesulfonate; MS-222) to an unused container and adding 150 ml of water to it, then mixing. We used a mesh screen to transfer the tadpole or froglet to the anesthetic bath, and allowed the Tricaine-S to take effect, anesthetizing the tadpole. Once the tadpole was unconscious, we recorded its Gosner stage and transferred it to a paper towel, then patted it with the paper towel to remove excess water and measured its snout to vent length (SVL) using a Mitutoyo® digimatic micrometer. We used a hypodermic needle to puncture the skin, and collected the tadpole's blood from the ventral abdominal vein using a microhematocrit capillary tube. We transferred the blood to a 100 µl Hamilton™ syringe to find the amount of blood collected, and then put the blood in a small snap-top microcentrifuge tube. Phosphate buffered saline (PBS) was added to the tube to bring the total volume of the sample to 120 µl. The tadpole was then euthanized in a lidded container with 70% Et OH. The blood sample was vortexed, placed in a microcentrifuge tube rack, and frozen until needed for the bacterial killing assay. This procedure was done for all of the 105 experimental tadpoles between August 10 and September 3, 2015. On September 3, 2015, we began the bacterial killing assays. We added 10 µl of the stock *A. hydrophila* solution (non-diluted) to each blood sample tube, and vortexed the tubes. We put 120 µl of PBS in a separate microcentrifuge tube and added 10 µl of the stock *A. hydrophila* solution to act as a control for the assay. Then, we incubated all of the tubes at 27˚C for 30 minutes. While the samples incubated, we used a Finnpipette™ multichannel pipette to transfer 100 µl of tryptic soy broth to each row of wells on a polystyrene 96-well plate.

After the incubation period was completed, we added 50 µl of the blood samples and bacterial inoculate mixture to each well, so that each tadpole blood sample was assayed in duplicate wells. However, two of the wells contained only the control PBS/bacteria solution to act as blanks, allowing us to determine background optical density. After filling each well as described, 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.

STATISTICAL ANALYSIS

We used general linear mixed models with *A. hydrophila* killing ability as the dependent variable; treatment group as the independent variable; 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 with treatment group (control, pathogen, acid, acid and pathogen) when considering varying Gosner stages, SVL and sample freeze times for the tadpoles. We had a random variable of container number within treatment group, because not all of the tadpoles were housed in the same container in each treatment group, and some containers within treatment groups could have had slightly different environments than others (e.g. more buildup of mucus on the sides of the containers, more available oxygen in those containers that had fewer tadpoles as a result of die offs).

We also ran a mixed model with SVL as the dependent variable, treatment group as the independent variable and Gosner stage as a covariate to determine whether SVL (a measure of growth) differed with treatment group when considering varying Gosner stages.

All statistical analyses were completed using SPSS. We considered a p-value of less than 0.05 to be statistically significant.

RESULTS

We found that there was no significant interaction between treatment group and Gosner stage ($F_{3,89}$ = 1.066, P = 0.368), treatment group and SVL ($F_{3,89}$ = 1.460, P = 0.231) or treatment group and sample freeze time ($F_{3,89}$ = 1.335, P = 0.268) with regards to *A. hydrophila* killing ability. Because there were no significant interactions, we looked at main effects. We found that *A. hydrophila* killing ability did not differ significantly by treatment group, though there was a strong trend toward lowered killing ability in each of the three treatment groups compared to the control group ($F_{3,89}$ = 2.447, P = 0.069; Fig. 1). We also found no significant covariance between *A. hydrophila* killing ability and Gosner stage ($F_{1,89}$ = 0.000, P = 0.992), *A. hydrophila* killing ability and SVL ($F_{1,89}$ = 0.072, P = 0.789), or *A. hydrophila* killing ability and freezing time of each tadpole blood sample ($F_{1,89}$ = 0.297, P = 0.587). However, we did find that the random variable of container number within treatment groups did explain a significant amount of variance in *A. hydrophila* killing ability of *O. septentrionalis* tadpoles (Wald Z = 6.782, P < 0.000), which essentially validated our statistical control for container effects. While our observed *A. hydrophila* killing ability among treatment groups was not statistically significantly different, the trend towards higher *A. hydrophila* killing ability in control *O. septentrionalis* tadpoles is likely biologically significant (P = 0.069).

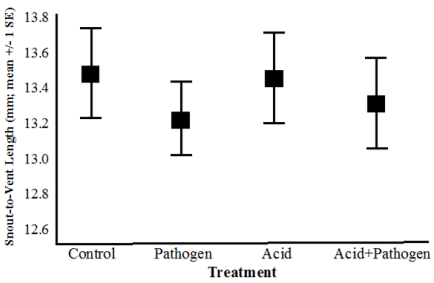

Fig1. *The effect of exposure to Aeromonas hydrophila, acidified water, or a combination of those stressors, on A. hydrophila killing ability (BKA) in Osteopilus septentrionalis tadpoles. A. hydrophila killing ability did not significantly differ between treatment groups ($F_{3,89}$ = 2.447, P = 0.069).*

We found that there was no significant interaction between treatment group and Gosner stage with regards to SVL in *O. septentrionalis* tadpoles ($F_{3,97}$ = 0.415, *P* = 0.743). Because there was no significant interaction, we considered the main effects, and found that SVL did not differ significantly by treatment group ($F_{3,97}$ = 0.416, *P* = 0.742; Fig. 2). There was no significant covariance between SVL and Gosner stage ($F_{1,97}$ = 1.663, *P* = 0.200).

DISCUSSION

Our hypotheses were that water acidification would have a negative impact on the immune defense of *O. septentrionalis* tadpoles, and that multiple stressors (acid and *A. 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 acts as an immune disruptor in northern leopard frogs. Because acid acts as both a physiological stressor and an immune disruptor, we thought that acid would decrease immune defense, and that the added presence of a pathogen stressor in acidic conditions would decrease tadpole immune defense, as measured by *A. hydrophila* killing ability, even more than acid alone. The hypothesis that acid would decrease immune defense was not statistically supported in *O. septentrionalis* tadpoles (Fig. 1).

However, we did find a trend towards greater immune defense in control tadpoles, suggesting a possible biological relevance to our findings, though not statistically significant (Fig. 1). The hypothesis that multiple stressors would further decrease immune defense was not supported; we found no significant differences in *A. hydrophila* killing ability among non-control treatments (Fig. 1). Additionally, our hypothesis that acid exposure would cause decreased growth in *O. septentrionalis* tadpoles was not supported; we found that acid did not have a significant effect on growth, as measured by SVL (Fig. 2). Our results, though not statistically significant, suggest that the presence of stressors may decrease bacterial killing ability in this species, but the nature of the stressor is not as important.

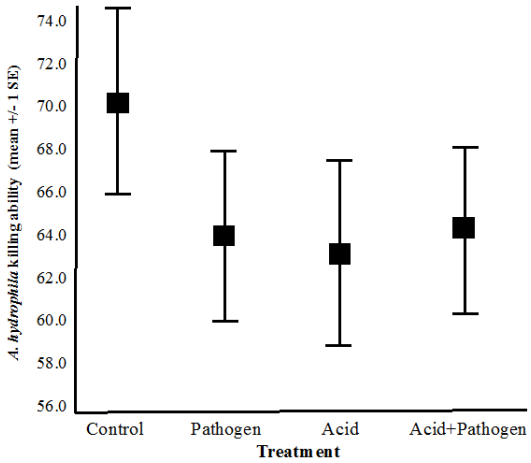

Fig2. *The effect of exposure to Aeromonas hydrophila, acidified water, or a combination of those stressors, on snout-to-vent length (SVL) of O. septentrionalis tadpoles. SVL did not significantly differ between treatment groups (F3,97=0.416, p=0.742).*

Ample evidence from other studies demonstrates the negative impact of freshwater acidification on vertebrate life (e.g., Schindler 1988; Räsänen et al. 2002; Vatnick et al. 2006; Miaud et al. 2011), therefore; we would caution against any conclusion that this study demonstrates a completely neutral effect of acidification on freshwater vertebrates. In the species examined in the aforementioned studies that demonstrate a negative effect of acidification on amphibian health and performance, the species tend to have a longer larval period, which may result in differential impacts of acidification on the tadpoles. Further, each of those studies utilized different measurements of physiological condition than did we, and as such, if they had studied innate immune responses against this known pathogen, they may have seen similar results. Conversely, a source of discrepancy between our hypotheses and our results for the

O. septentrionalis tadpoles could be due to unique qualities of our study species itself. We initially experimented on *O. septentrionalis* tadpoles because they were easy to obtain, and egg masses were available at the beginning of the experiment. However, Cuban tree frogs are an invasive species in Florida, and they have been shown to have a higher tolerance to salinity and other stressors than native species (Brown and Walls 2013). In addition to increased tolerance to salinity, Cuban tree frogs have a strong tolerance for tail damage during the larval stage (Koch and Wilcoxen 2018), tolerance of temperatures lower to those experienced within their normal range (Going and Wilcoxen 2018), and tolerance of the herbicide atrazine (Rohr et al. 2013). Rohr et al. (2013) did find that exposure to *Batrochocytrium dendrobatidis*, a pathogenic fungus, and atrazine early in the larval period has a long-lasting negative impact on survival in Cuban tree frogs. *Batrochocytrium dendrobatidis* is considered an emerging infectious disease among amphibians, and far less is known about mechanisms underlying susceptibility among individuals in populations; whereas, *A. hydrophila* (the pathogen used in our study) has long been considered an opportunistic bacterium, with its presence on the skin or in the body of tadpoles only manifesting as disease in times of elevated stress, poor nutritional condition, or decreased immunocompetence. With the exception of the negative effects of atrazine and *B. dendrobatidis* on Cuban tree frogs, many of these other studies have found this species to be resilient to stress, therefore; it is possible that Cuban tree frogs have thrived as an invasive species because they are more resilient to stressors and, in general, less sensitive to environmental disturbances, such as acidification and pathogen threat, than native species.

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