

Fertilizer exposure affects *Hyla versicolor* (gray tree frog) larval growth and
development

BIOS 35502-01: Practicum in Field Biology

Rebecca Flynn

Advisor: Matt Michel, Ph.D.

2009

Abstract

Amphibian populations worldwide are experiencing mass extinctions, the causes of which have yet to be fully determined. Portions of this have been linked to addition of mineral nitrogen to water bodies in which amphibians breed. This study set out to determine whether two types of nitrogen fertilizer addition to the water column—that is spills and run-off—differentially affect larval growth, development, activity, and survivorship. Concentrations of 10 mg/L of granular urea were added as pulses—simulating one-time large spills—and presses—simulating run-off—to pools containing *Hyla versicolor* tadpoles. Survivorship and activity did not differ among treatments. However, growth and development were both affected by the fertilizer: growth rate was higher in fertilizer treatments than in control, and the stage of development was higher in fertilizer treatments than in control ones. Except for one day, there was no statistically significant difference between press and pulse treatments. These results indicate that previous studies' results can be applied to both fertilizer spill and run-off situations. In addition, it shows that fertilizer may not always be harmful to tadpole growth and development if added to the water column during the larval stage, which may have agricultural and wildlife management implications.

Introduction

Worldwide, amphibians have been undergoing a mass extinction event, caused by a variety of factors (Stuart et al. 2004). The class of declines that most

rapidly leads to extinction, termed “enigmatic decline,” has been the most difficult with respect to species conservation because the causes are still being determined and the only method for preservation is captive breeding, which does not always work for amphibians (Stuart et al. 2004). A portion of enigmatic decline has been linked to an increase in the amount of mineral nitrogen entering the water (Rouse et al. 1999). This increase comes from the use of fossil fuels, fixation of N₂ in fertilizer and other anthropogenic causes (Vitousek et al. 1997). Bodies of water become polluted with nitrogen from agricultural run-off, livestock, and industrial and human waste (Rouse et al. 1999). Such nitrogen pollution causes soil, stream, and lake acidification in many regions as well as a hastened loss of biodiversity (Vitousek et al. 1997).

The effects of nitrogen pollution on amphibians have been studied at length in recent years. Nitrite fertilizer has been shown to reduce wood frog (*Rana sylvatica*) hatching success as well as, at lower (0.5 mg/L NO₂) concentrations, cause mortality in early larval stages of both *R. sylvatica* and tiger salamander (*Ambystoma tigrinum tigrinum*) (Griffis-Kyle 2005). Their vulnerability to the toxic effects of nitrite decreased shortly after they hatched, showing differential effects of nitrite based on amphibian life stage (Griffis-Kyle 2005). However, nitrite exposure did slow both embryonic and larval development in those species, in addition to various other sublethal effects including decreased embryonic and larval growth and development rates (Griffis-Kyle 2005). Exposed embryos of

both species also hatched prematurely, reducing their oxygen consumption efficiency (Griffis-Kyle 2007).

Exposure to high concentrations of ammonium nitrate fertilizer (13.6-39.3 mg/L NO₃-N) over a short period of time (96 h) causes higher mortality than exposure at lower concentrations (2.5, 5, and 10 mg/L NO₃-N) maintained over longer periods of time (100 d) (Hecnar 1995). In both situations, exposure causes weight loss, reduced activity and physical abnormalities but the time until onset of these effects varies by treatment—tadpoles in acute treatments experience them earlier than those in chronic treatments (Hecnar 1995). This study additionally demonstrated that at concentrations often surpassed on agricultural land, test species experienced toxic effects (Hecnar 1995). *R. sylvatica* tadpoles exposed to ammonium fertilizer hatched earlier, were shorter in length upon hatching, and had a higher incidence of deformities at high concentrations of ammonium (Griffis-Kyle and Ritchie 2007). In addition, amphibian larvae exposed to nitrogen fertilizer are more susceptible to predation, possibly due to slowed growth (Griffis-Kyle and Ritchie 2007). Not only predator-prey but also competitive interactions are affected by increasing nitrogen concentrations. Ammonium nitrate exposure caused reduced survival for the American bull frog (*Rana catesbeiana*) when in competition with the American green frog (*Rana clamitans*); however, *R. clamitans* were heavier in mesocosms with nitrate added than in those without (Smith et al. 2006). These results show that amphibian

larval performance in competitive environments can be affected by nitrate pollution in both positive and negative ways (Smith et al. 2006). Granular urea fertilizer used in forest fertilization is lethal to some adult amphibians in high concentration doses, such as 450 kg N/ha, but less so in lower concentrations, such as 225 kg N/ha (Marco et al. 2001).

In natural settings, fertilizer is introduced to an ecosystem in two ways: as a one-time spill or as a constant leak, such as run-off from a nearby agricultural field. Granular urea is used in forest settings and distributed using airplane fly-overs (Marco et al. 2001), so it may enter a waterbody where tree frogs, such as *H. versicolor*, breed in both ways. Even though nitrogen is introduced in these two ways, no previous research has examined how differences in timing and amount of nitrogen exposure affect amphibian larval growth and development. This study is one of few to test how these two ways of adding fertilizer to a system will affect amphibian health. The specific hypothesis being tested is whether there is a difference in the effects of granular urea fertilizer on gray tree frog (*Hyla versicolor*) tadpole growth, development, activity and survivorship when added as a pulse—a large spill—versus as a press—a constant input—over the time of their development. It is expected that the pulse tadpoles will experience a higher mortality than the press tadpoles since the larger dose of fertilizer earlier in development, when the tadpoles are more vulnerable, will have a more deleterious effect than smaller doses consistently through development

(Griffis-Kyle 2005; Hecnar 1995). In addition, both fertilizer treatments will likely cause higher mortality than the control. With respect to growth, development and activity, both fertilizer treatments should reduce them, but likely the pulse more so initially while the effects appear in the press later (Hecnar 1995). As this study simulates realistic conditions of fertilizer introduction, it is an important step in illuminating amphibian response in their natural habitats.

Materials and Methods

Collection

On one night, during their breeding season, five *H. versicolor* amplexing pairs were caught at Vernal Pond 9 on the property of the University of Notre Dame Ecological Research Center (UNDERC) and brought back to a laboratory to breed. Egg masses were collected and stored in pools to develop into tadpoles.

Tank environments

Tadpoles from the pairs were assorted into pools at random. Forty tadpoles (eight from each of the five egg masses) were placed in each of 21 tanks of 0.5 m radius tanks filled to a depth of 0.14 m. The total volume of each tank was 0.11 m³ (110 L). All simulated pond environments also had 2 L water from a nearby vernal pond, 5 g of ground Pet Rabbit Food (Hartz NutritionTM), 100 g of leaf litter—composed approximately 70% of *Acer saccharum*, 20% *Populus tremuloides*, and 10% of grasses, twigs, and other plant material. Each tank was covered with a screen to prevent insect predators from laying eggs into the tank.

The addition of fertilizer was manipulated in a press or pulse method to give a total of three treatments, each with seven replicates: control, pulse, and press. The assignment of pool treatments was randomized. To carry out the pulse, 2391 mg of urea fertilizer (©The Andersons, Inc.) were added at once, two days after tadpoles were added to their pools. For the press experiments, 66-67 mg were added twice a day for 18 days (days 2 through 19), beginning the same day as the pulse. The total concentrations for each treatment were similar: 10 mg/L (Griffis-Kyle and Ritchie 2007). The fertilizer was dissolved in approximately 50 ml of water before addition to the pools. The control tanks received no fertilizer.

Data collection and Analysis

Throughout the experiment, observations and measurements of the tadpoles' growth, development, and activity were taken. At the experiment's end, survivorship was determined. Growth was measured by mass, taken every day, except day 11, from five tadpoles, haphazardly caught from each pool. Beginning on day 17, development was measured using the stages described by Gosner (1960). Both growth and development data was collected until 4 days after fertilizer treatments stopped. Activity readings were taken as a proportion of how many tadpoles were seen active in the pool over how many tadpoles were seen. These data were collected every day, except days 8 and 11, beginning the day before fertilizer was added until 3 days after fertilizer addition ceased (day 22). Data was also collected on deformity prevalence.

Data analyses were conducted using Levene's test for homogeneity of variance and Kolmogorov-Smirnov test for normality. One-way ANOVA tests were run for survivorship and activity to find whether there was a statistically significant difference between those factors given treatment type. To determine whether the masses and developmental stages of tadpoles had changed differentially by treatment over the 23 days of the experiment, Repeated Measures ANOVAs were run of treatment versus mean mass or mean developmental stage from each pool on a given day. Some Bonferroni post-hoc analyses were used on growth and development data as well. These statistical tests were run using SYSTAT 12.0 software package (Systat Software, Chicago, IL).

Results

Growth

A Levene's test showed that the variance of the data was indeed homogeneous (p -values >0.05). The Repeated Measures ANOVA indicated a statistically significant difference between treatments ($df=2$, $F=17.92$, $p<0.001$). Tadpoles in control treatments were 73.2% and 70.2% the size of those in press and pulse treatments, respectively. Growth was affected by time as well, with all tadpoles in all treatments growing over time ($df=20$, $F=106.78$, $p<0.001$). In addition, there was a statistically significant interaction term between the treatment and the growth over time, indicating that tadpoles in different treatments grew at different rates ($df=40$, $F=5.23$, $p<0.001$). Those exposed to

fertilizer, in both press and pulse treatment methods, grew at higher rates than tadpoles in control pools (Fig. 1).

ANOVAs were run for each day to determine when the treatments diverged. The p-values became statistically significant ($p < 0.05$) beginning on Day 12 and remained so through Day 23. There was no statistically significant difference between pulse and press treatments overall, except on day 18, the penultimate day of press treatments, when pulse treated tadpoles were found to be larger than those of both press and control and press larger than control, according to a Bonferroni post-hoc analysis (Press/Pulse $p = 0.0374$, Pulse/Control $p < 0.001$, Press/Control $p < 0.001$).

Development

A Levene's test showed homogeneity of variance for the data (p -values > 0.05). Developmental stage changed as a function of time ($df = 6$, $F = 97.76$, $p < 0.001$) and as a result of treatment type ($df = 2$, $F = 40.79$, $p < 0.001$) but there was no statistically significant interaction term ($df = 12$, $F = 0.96$, $p = 0.494$). On all days, the tadpoles exposed to fertilizer were at higher developmental stages, with no difference between pulse and press, than those in control treatments, but the rate of stage progression did not vary between treatments (Fig. 2). The final mean developmental stage of control tadpoles was 96.6% and 96.1% that of press and pulse treated tadpoles, respectively.

ANOVAs run for each day showed statistically significant p-values

($p < 0.05$). A Bonferroni post hoc test was conducted for day 18 that showed statistical significance between developmental stages in pulse versus press pools as well as between control and both fertilizer treatments (Control/Pulse $p < 0.001$, Control/Press $p = 0.001$, Pulse/Press $p = 0.022$). The tadpoles in pulse pools were at higher stages of development than those in press and control pools.

Activity

The overall averages for each pool were normally distributed, based on a Kolmogorov-Smirnov test, and homogeneous of variance, based on Levene's test. An ANOVA was also run of the overall mean activities of tadpoles against treatment type. It indicated no statistically significant difference ($df = 2$, $F = 0.238$, $p = 0.791$). Tadpole activity was unaffected by fertilizer treatment (Fig. 3).

Survivorship

Since survivorship was only able to be determined at the experiment's end, an ANOVA was run comparing survivorship between pools of each treatment type. The data was normally distributed (K-S test $p = 0.648$) and homogeneous in variance (Levene's test $p = 0.482$). There was no statistically significant difference in survival between treatment types ($df = 2$, $F = 0.0264$, $p = 0.974$), indicating a standard mortality—all treatments types experienced similar numbers of deaths (Fig. 4).

Other Observations

A small number, approximately 5, tadpoles in all fertilizer treatment pools

experienced effects of ionic imbalances by either bloating or shriveling. No tadpoles in control pools experienced either.

Discussion

Though some tadpoles were introduced to a chemical agent, the mortality of the exposed tadpoles did not differ from those that were unexposed. Though unexpected, it is consistent with the finding that vulnerability to nitrogen fertilizers decreases as amphibian development progresses (Griffis-Kyle 2005). The tadpoles had all grown and developed for a few weeks before exposure. Therefore, as survivorship among treatments was similar, the possibility of reduced competition for food can be eliminated as a source of the differential growth and development experienced by those tadpoles exposed to fertilizer and those not.

Contrary to expectation, activity was not affected by fertilizer treatments. Many studies of nitrite, nitrate, and ammonium fertilizers had shown that activity decreases due to fertilizer exposure (Hecnar 1995), but in this study it was unaffected. Nitrogen fertilizer, specifically urea, seemed to enhance tadpole growth and development, rather than decrease fitness, as was expected. Tadpoles exposed to both fertilizer treatments grew faster and reached higher developmental stages in the time span of this study. Nitrogen as a nutrient has been shown to promote tadpole growth (Peacor 2002). A positive effect of nitrate fertilizer was found on *R. clamitans* tadpoles, especially when only experiencing

intraspecific competition, likely due to an increase in the availability of periphyton (Smith et al. 2006). Therefore, as urea fertilizer is also rich in the nutrient nitrogen, it likely increased the available nutrition for the *H. versicolor* tadpoles, thereby promoting growth. The growth effect was not evident until eleven days after the fertilizer treatments began, suggesting that increasing nutrient availability was gradual. However, introducing this additional nutrition all at once versus in smaller amounts over time did not make a difference. The one day on which it did seem to make a difference, day 18, is likely anomalous, caused by catching more larger tadpoles at higher developmental stages and fewer of the smaller, lower developmental stage tadpoles in pulse pools than in press ones.

This study could be repeated with fewer tadpoles overall or weighing and staging a higher proportion of them to determine the source of the anomaly and to potentially provide a more precise result. In addition, if the study had been performed over a longer period of time, the effects of fertilizer addition may have become more pronounced for each treatment, potentially differentiating between the two fertilizer treatments since the last press treatments were added only four days before the experiment's end. Between days 17 and 23, tadpoles developed at the same rate with those in fertilizer pools at higher stages; therefore, they must have developed more rapidly earlier. Staging should be done from the beginning of the study, instead of begun near the end, to determine when in development the

tadpoles exposed to fertilizer were developing at a higher rate.

Other future studies could look at whether the affects of pulse versus press fertilizer treatments have differential effects if the fertilizer is added earlier, for example, during the embryonic stages, when they are more vulnerable. In natural settings, fertilizer isn't introduced only after development has progressed past those stages, so run-off and fertilizer spills would affect embryos too. Maybe differential survival, activity, and growth and development would be seen in such cases. Another possibility is that *H. versicolor* tadpoles are more resilient to fertilizer toxicity and sublethal effects than other amphibians, as *R. clamitans* were more resilient than *R. catesbeiana* (Smith et al. 2006), so this study should be modified and conducted using other test species.

Even though it appears that fitness of tadpoles increases with fertilizer addition during the larval stages, more studies are necessary to determine the effects when exposed to predation or higher competition. Since amphibian larvae are more susceptible to predation when their growth is diminished by fertilizer treatments (Griffis-Kyle and Ritchie 2007), a possible consequence of fertilizer added after larval development begins is less predation upon the tadpoles with enhanced growth. If such a phenomenon occurs, tadpole predators may experience a decline in survival and the ecosystem at large may experience a change in nutrient flow. If ecosystems are not harmed by such low concentration fertilizer additions after this stage of development, agriculture and forest

management programs could use this understanding and refrain from fertilizer addition during and soon after the breeding season of amphibians and add fertilizer later on without detrimental effects.

The most important result of this study is that it legitimizes conclusions drawn from previous studies conducted on the effects of fertilizer on tadpole development. The results of those studies can be applied to situations in which either a fertilizer spill or run-off occurs since the effects are the same. Results of those studies can be used in considerations of fertilizer utilization in agriculture, forest management, wildlife management, and policy-development.

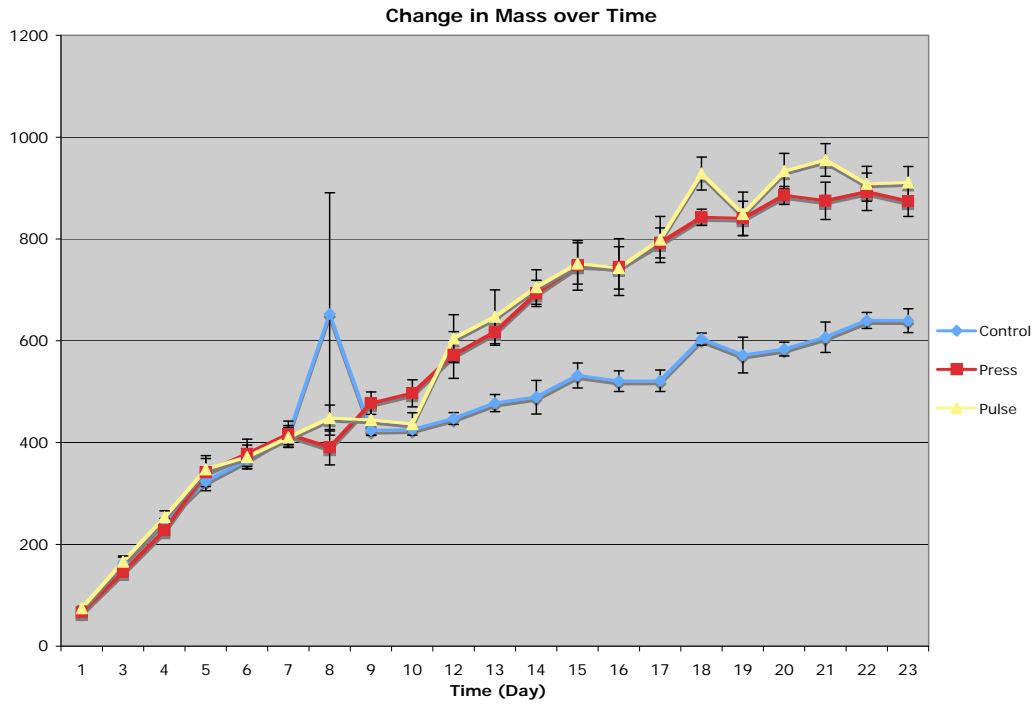
Figures

Figure 1. Change in tadpole mass over time for each of the three treatment types.

Control tadpoles are indicated by the line with diamond points, press ones by the line with square points, and pulse ones by the line with triangular points. Vertical lines indicate one standard error.

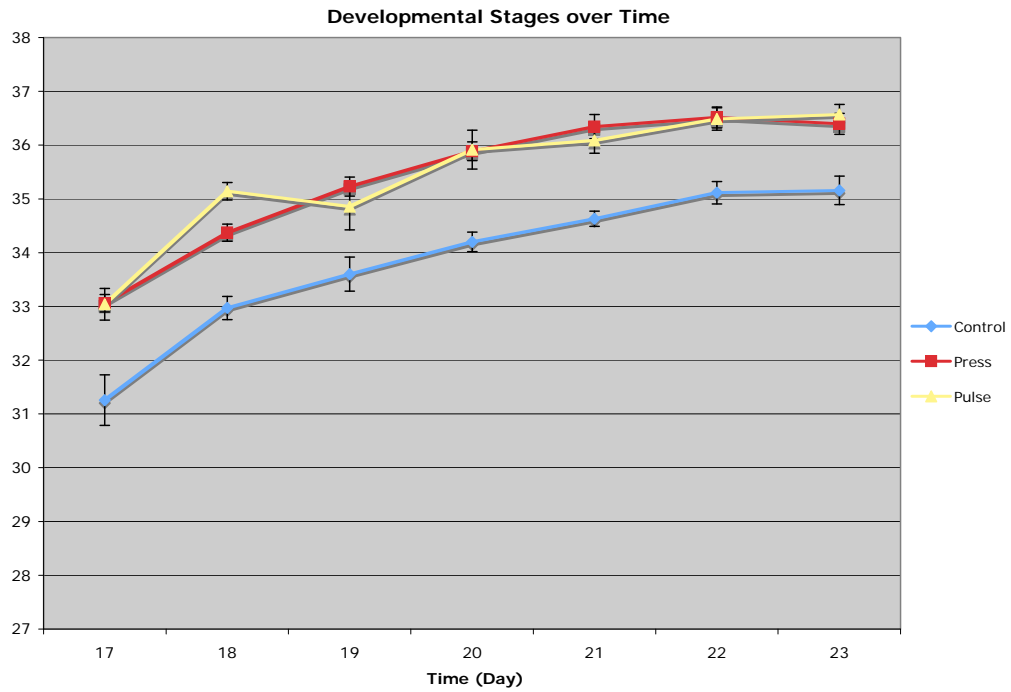


Figure 2. Change in developmental stage (Gosner 1960) between day 17 and day 23. Control tadpoles are indicated by the line with diamond points, press ones by the line with square points, and pulse ones by the line with triangular points. Vertical lines indicate one standard error.

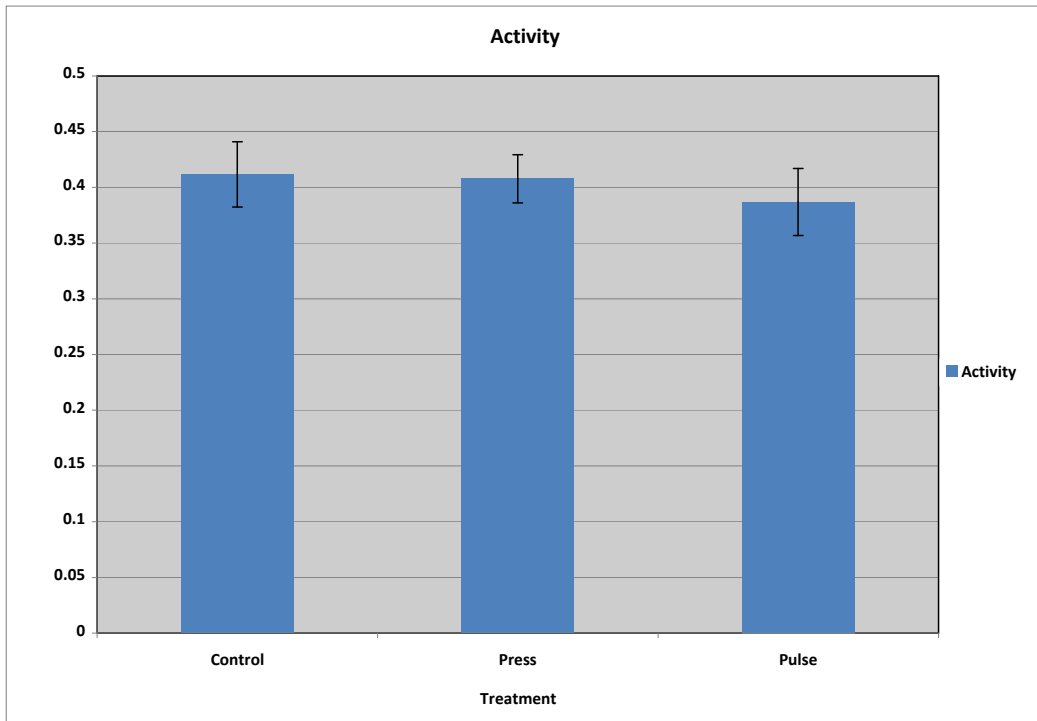


Figure 3. Overall activity of tadpoles by treatment type. Lines indicate one standard error.

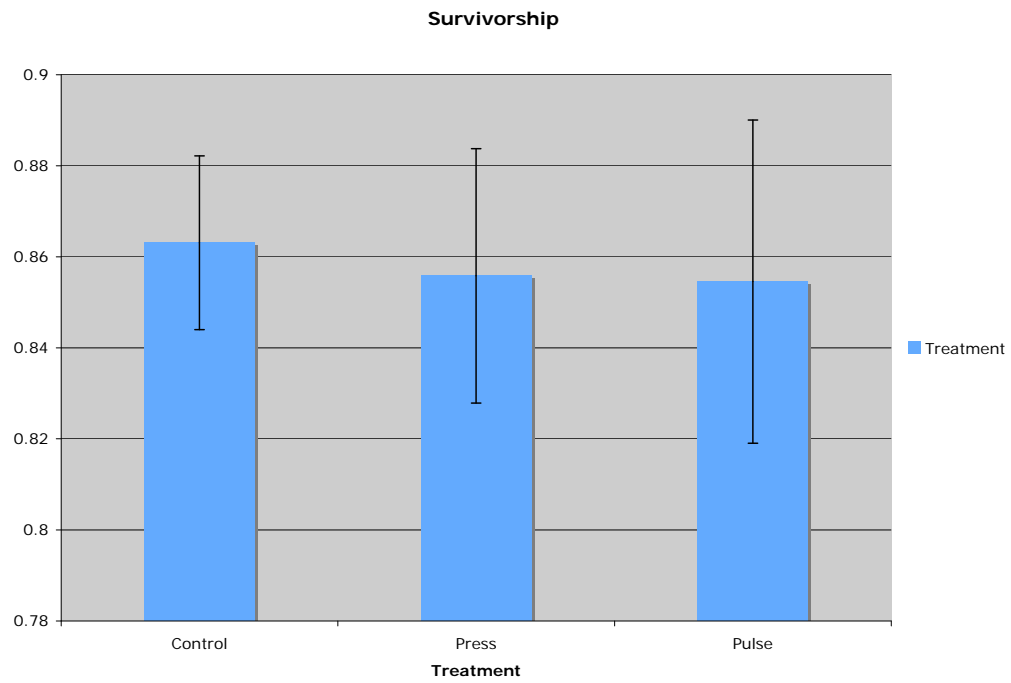


Figure 4. Survivorship for each treatment type over the entire course of the experiment. The lines indicate one standard error.

Acknowledgements

I wish to thank the University of Notre Dame for the opportunity to conduct this research and for providing the use of all facilities and necessary materials necessary at their Ecological Research Center. I wish to also thank the Hank family for funding this research, course, and the upkeep of all the facilities utilized through The Bernard J. Hank Family Endowment. On more personal notes, I also thank my mentor, Matt Michel, for all of his suggestions, guidance and assistance throughout the entire process from proposal to final draft edits. I am also grateful to our lab technician, Heidi Mahon, for her help with statistics. In addition, I thank the following classmates for helping me on my project: David Cray, Alex Hall, Nathan Hammes, Grace Loppnow, Navit Reid, and Shayna Sura for their assistance with data collection as well as Ashley Bozell for her help proof-reading this paper. Without their help and the company of all the other students, I would have lost my sanity. Of these, I most need to thank David Cray for assisting me almost every night as I weighed and staged tadpoles; his help and emotional support were valuable beyond measure. Finally, I must thank the music that kept me going. I am very grateful for both the UNDERC playlist and those who contributed to it for keeping me positive and entertained during the long hours of work as well as for the soothing and beautiful music of the Laclede Quartet for helping me focus.

References Cited

- The Andersons, Inc. Urea. Ag Products Group. Maumee, OH.
- Gosner, Kenneth L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183-190.
- Griffis-Kyle, K. L. 2005. Ontogenic delays in effects of nitrite exposure on tiger salamanders (*Ambystoma tigrinum tigrinum*) and wood frogs (*Rana sylvatica*). *Environmental Toxicology and Chemistry* 24(6):1523-1527.
- Griffis-Kyle, K. L. 2007. Sublethal effects of nitrite on eastern tiger salamander (*Ambystoma tigrinum tigrinum*) and wood frog (*Rana sylvatica*) embryos and larvae: implications for field populations. *Aquatic Ecology* 41:119-127.
- Griffis-Kyle, K. L. and M. E. Ritchie. 2007. Amphibian survival, growth and development in response to mineral nitrogen exposure and predator cues in the field: an experimental approach. *Oecologia* 152:633-642.
- Hartz Nutrition™. Pet Rabbit Food. The Hartz Mountain Corporation. Secaucus, NJ.
- Hecnar, S. J. 1995. Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. *Environmental Toxicology and Chemistry* 14(12):2131-2137.
- Marco, A., Cash D., Belden L. K., and A. R. Blaustein. 2001. Sensitivity to urea fertilization in three amphibian species. *Archives of Environmental*

Contamination and Toxicology 40:406-409.

Microsoft Office Software. 2003. Microsoft Excel. USA.

Peacor, S. D. 2002. Positive effect of predators on prey growth rate through induced modifications of prey behaviour. *Ecology Letters* 5:77-85.

Rouse, J. D., Bishop C. A., and J. Struger. 1999. Nitrogen pollution: an assessment of its threat to amphibian survival. *Environmental Health Perspectives* 107:799-803.

Smith, G. R., Temple K. G., Dingfelder H. A., and D. A. Vaala. 2006. Effects of nitrate on the interactions of the tadpoles of two ranids (*Rana clamitans* and *R. catesbeiana*). *Aquatic Ecology* 40:125-130.

Stuart, S. N., Chanson J. S., Cox N. A., Young B. E., Rodrigues A. S. L., Fischman D. L., and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science* 306:1783-1786.

Systat Software. 2008. Systat 12. Chicago, Illinois.

Vitousek, P. M., Aber J. D., Howarth R. W., Likens G. E., Matson P. A., Schindler D. W., Schlesinger W. H., and D. G. Tilman. 1997. Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications* 7:737-750.