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Evaluation of Phosphate Toxicity in Cope's Gray Treefrog (*Hyla chrysoscelis*) Tadpoles

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ABSTRACT.—**Phosphate pollution is a widespread problem resulting from agricultural runoff and urban wastewater. Phosphates are known to cause eutrophication and algal blooms, but little is known about phosphate toxicity, particularly among amphibians. To investigate possible phosphate toxicity, *Hyla chrysoscelis* tadpoles were exposed to concentrations ranging from 0–200 mg/L PO_4-P for 15 days. Phosphate was found to have no effect on the survival, growth, or developmental stability of the tadpoles, indicating that phosphate may not be toxic to this species at levels associated with anthropogenic inputs. Phosphate was found to increase the pH of the test water, which, in conjunction with other stressors, may have negative effects within aquatic communities. However, phosphate could also affect anuran tadpoles positively by increasing algal food resources. Because phosphate is a ubiquitous pollutant, further testing using more complex experimental designs is warranted.**

Pollution from nutrients, such as phosphate, is widespread across the United States in the forms of point and nonpoint source pollution (Smith et al., 1987; Carpenter et al., 1998). The main causes of phosphate pollution are fertilizer runoff from agricultural fields (Cooke and Williams, 1973; Andraski and Bundy, 2003) and urban wastewater from detergents and sewage (Devey and Harkness, 1973). Although it has been established that phosphorus is often a limiting nutrient in freshwater habitats (Correll, 1998), the concentrations of phosphorus in polluted systems can exceed the levels that can be used by algae and macrophytes in aquatic systems (Wetzel, 2001). In such cases, phosphate is not immediately depleted by primary producers (Wetzel, 2001), creating the possibility of phosphate toxicity.

It is important to evaluate pollutants for their toxicity in order to determine risk management strategies (Connell and Miller, 1984). Little toxicological information is available for phosphate, and most of it involves human overdoses (i.e., Biberstein and Parker, 1985; Azzam, et al. 2004). However, two studies have examined the effects of phosphate on anurans and found that the presence of phosphate affected each species differently. Hamer et al. (2004) found that over 91 days (starting at Gosner stage 25) 15-mg/L phosphate increased survival in *Limnodynastes peronii*, decreased survival in *Litoria aurea*, and had no effect on *Crinia signifera*, whereas Smith (2007) found no effect of 20-mg/L phosphate over four days in *Rana sylvatica* at Gosner stage 26. More research on phosphate toxicity is needed to better understand this variation in response. Toxicological information is very important for amphibians, because they are particularly susceptible to pollution because of their sedentary nature (when compared to other vertebrates) and permeable skin and embryonic membranes (Duellman and Trueb, 1994; Carey and Bryant, 1995). Additionally, habitat degradation through pollution is considered one of the underlying causes of global amphibian declines (Diana and Beasley, 1998; Semlitsch, 2003).

Measures of developmental stability (DS), the ability to buffer stresses during development, could provide important tools for mitigating amphibian declines (Alford et al., 1999). Scientists can use DS, often measured as fluctuating asymmetry (FA), as an early warning system to detect stress in a population before that stress has negative fitness consequences (Parsons, 1992; Clarke, 1995). FA examines the frequencies within a population of normally distributed minor deviations from perfect bilateral symmetry (Palmer, 1994; Møller and Swaddle, 1997; Hoffman and Woods, 2001). This type of measure could potentially be integrated into population monitoring protocols (Alford et al., 1999). To use FA as a monitoring tool, it is important to know what types of stresses, such as toxicity, induce high levels of FA. Thus, we conducted a standard 15-day toxicity test (Clesceri et al., 1989) to determine the effects of phosphate on the survival, growth, and

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developmental stability of Cope's Gray Treefrog (*Hyla chrysoscelis*) tadpoles.

Materials and Methods

Hyla chrysoscelis eggs were collected on 2 June 2006 from a pond at Hancock Biological Station in Calloway County, Kentucky. Eggs were collected from three spatially separated regions within the pond; thus, the eggs were assumed to originate from separate amplecting pairs. Eggs were placed in a very soft (pH = 6.4–6.8, hardness = 10–13 mg $CaCO_3$/L) reconstituted freshwater solution (Clesceri et al., 1989) and hatched in the lab. All eggs hatched two days after collection. The experiment was started three days after hatching, when tadpoles, ranging from stage 23–25 (Gosner, 1960), were placed in glass culture dishes containing 100 ml of very soft, reconstituted freshwater (as above) and randomly assigned to one of five treatments: 0, 1, 10, 100, and 200 mg/L PO_4-P as sodium phosphate. Concentrations were not chosen to be environmentally relevant (Chow-Fraser et al., 1998) but rather to provide baseline toxicity information on phosphate. For reference, phosphate concentrations have been found as high as 15.5 mg/L (Chow-Fraser et al., 1998) but are naturally less than 0.03 mg/L (EPA, 2003). No sodium control was used, because sodium has been found to have no effects on tadpole growth or survival at concentrations of sodium chloride equivalent to the sodium concentration of the 100-mg/L treatment (Baker and Waights, 1994). Each treatment had 40 replicates with one tadpole per dish as a replicate. Each day, the number of dead tadpoles was assessed. The water was changed every three days to produce a static renewal experiment (Clesceri et al., 1989), and water samples were taken from a randomly chosen dish from each treatment at the time of the water change. On days when the water was not changed, 10 ml of the very soft water solution with 0 mg/L PO_4-P was added to every dish to compensate for water loss resulting from evaporation. In one randomly chosen dish from each treatment, the temperature to the nearest 0.25°C, pH to 0.01, and dissolved oxygen to 0.01 mg/L, were measured every one to two days using a mercury thermometer, Orion pH meter model 210A, and YSI dissolved oxygen meter model 54A, respectively. Tadpoles were fed ad libitum a mixture of ground alfalfa pellets and fish food at a 3 : 1 ratio.

The experiment lasted for 15 days, approximately half to three-fourths of the total larval period of *H. chrysoscelis* under similar laboratory conditions (Earl and Whiteman, 2009). On the final day, tadpoles were weighed, photographed, and sacrificed. Mass was recorded to the nearest 0.01 g. Tadpoles were positioned and photographed three times each to assess measurement error (unpubl. data) using a Pixera model PVC 100C through a microscope at a 50× magnification. All photographs were taken with a resolution of 1,280 by 1,024 pixels. Photographs were measured in pixels for eye width, the distance from the eye to the nare, and snout–vent length (SVL) using the software ImageJ (NIH, 2006). All measurements were taken without knowledge of previous measurements to prevent bias.

A fungus consistent with *Saprolegnia* (Whitaker and Wright, 2001) was found growing on the food in the dishes. *Saprolegnia* is common in freshwater (Whitaker and Wright, 2001), and it was likely introduced to the lab after collection of eggs in pond water. To assess the impact of the fungus on the experiment, each dish, on day 14, was categorized into one of three levels of fungus: (1) no fungus apparent; (2) small amounts of fungus on food; and (3) mats of fungus on food. Additionally, two tadpoles, one from the control and one from the 10-mg/L treatment, were found on day nine with the fungus covering their mouthparts with mycelial strands hanging down from their oral disk. Both tadpoles were dead the next day and were excluded from the survival analysis. A chi-squared test of independence was performed to determine whether the amount of fungus differed among treatments. All dishes with the highest level of fungus were excluded from further analysis. Additionally, in all analyses except in the survival analysis, fungus level (1 or 2) was used as a factor to partition out variation caused by fungal presence.

All statistical analyses were performed using PC SAS. Survival analysis was performed using the Kaplan-Meier test (proc LIFETEST) to test for differences among treatments in survival through time (Allison, 1995). Because the strata statement only tests whether all treatments have the same survivor function (Allison, 1995), the analysis was repeated using all possible combinations of pairs of treatments to determine which pairs of treatments were significantly different (Earl and Whiteman, 2009). The alpha value was adjusted using the Bonferroni correction, making it 0.005 (Hochberg, 1988). Because the fungus was not assessed until day 14, it could not be used as a covariate in the survival analysis. One-way ANOVAs (proc GLM) were performed on all physical/chemical data to determine differences between treatments, and two-way ANOVAs (proc GLM) were performed on all response variables (mass, SVL, and body condition [mass/SVL]) to determine the effects of phosphate and fungus. Prior to analysis, SVL

was assessed for measurement error using a one-way ANOVA on the measurements of three photographs per individual with individual as a random effect. If measurement error was not significant, the three measurements of SVL were averaged for each individual. For all ANOVAs performed, homoscedasticity was checked by plotting the residuals against the predicted values of the model. All models showed homogeneity of variance.

For developmental stability, the traits of eye width and the distance from eye to nare were tested for adherence to the following assumptions: low measurement error, no directional asymmetry, and no size dependence before analysis, as is the convention before FA analyses (Palmer, 1994). Measurement error was assessed with a two-way ANOVA (proc GLM) on the measurements of the three pictures for each individual using individual as a random effect and side (left or right) as a fixed effect. The significance of this interaction indicated that the between-sides variation was greater than the measurement error; thus, measurement error was low enough for FA analysis (Palmer, 1994). This analysis also assessed directional asymmetry as a significant side effect. Assuming low measurement error, the three measurements were averaged for each side for each individual. The left side was subtracted from the right side for each individual for each trait to produce an asymmetry index (Palmer, 1994). A simple linear regression was performed on the absolute value of the asymmetry index using average trait size as the explanatory variable to determine size dependence of FA. If all assumptions were met, a two-way ANOVA was performed on the absolute value of the asymmetry index to determine differences among treatments and fungal levels in FA. For traits with significant directional asymmetry, an ANOVA on the signed asymmetry was performed to determine whether the degree of directional asymmetry varied among treatments and fungal level. Additionally, the distribution was centered by subtracting the mean asymmetry from all points to correct for directional asymmetry, creating a new asymmetry index. The absolute value was taken of the corrected asymmetry index and analyzed using an ANOVA to assess differences among treatments and fungal levels in FA (Palmer, 1994). Traits with directional asymmetry were assessed for treatment differences in directional asymmetry (signed asymmetry) and fluctuating asymmetry (unsigned, centered asymmetry), because recent studies have found that stressors can affect either directional or fluctuating asymmetry (Lens and Van Dongen, 2000; Earl and Whiteman, 2009). Statistics on all measurements from image analysis used pixels as the unit of measure, but for comparison with other studies, pixels can be converted to millimeters by multiplying by 0.0087. Means are given ± 1 SE.

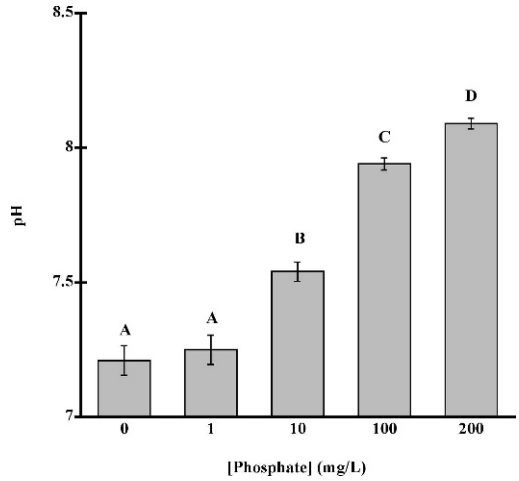

FIG. 1. Mean pH (± SE) for different concentrations of phosphate. Treatments with different letters are significantly different using the Tukey pairwise comparison.

RESULTS

Water samples taken from dishes revealed phosphate concentrations close to target levels, except on day 11, when the concentrations were elevated in all treatments. At this time, the control exceeded 1 mg/L PO_4-P, but no other treatments overlapped in concentration. There was no difference in dissolved oxygen ($F_{4,45}$ = 0.84, P = 0.50; 7.46 ± 0.10 mg/L) or temperature ($F_{4,55}$ = 0.15, P = 0.96; 22.46 ± 0.08°C) among the treatments. pH differed significantly across treatments with an increase in pH with phosphate concentration ($F_{4,55}$ = 106.63, $P < 0.0001$, Fig. 1). The amount of fungus present in the dishes differed significantly among treatments. The control and lower concentrations had significantly more fungus than the 200-mg/L treatment (χ^2_8 = 91, $P < 0.0001$, Fig. 2).

The Kaplan-Meier survival test revealed an overall significant difference among treatments (χ^2 = 12.54, P = 0.01), but survival did not increase or decrease with the concentration of phosphate (Fig. 3). No mortality occurred in the 1-mg/L and 100-mg/L treatments, and the control had the lowest survival (Fig. 3).

SVL had very low measurement error ($F_{185,372}$ = 104.18, $P < 0.0001$). Tadpoles from the control and 200-mg/L treatments were smaller in SVL ($F_{4,155}$ = 2.80, P = 0.03; Fig. 4A) and body condition ($F_{4,155}$ = 2.26, P = 0.06; Fig. 4B) than

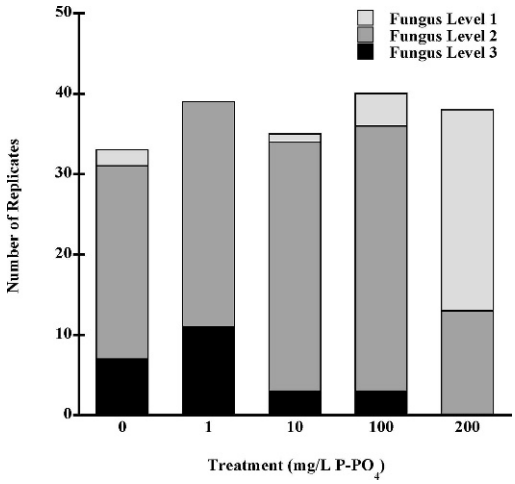

FIG. 2. Number of replicates in each fungus level by treatment. (Fungus level 1: no fungus apparent, 2: small amounts of fungus, 3: fungal mats.)

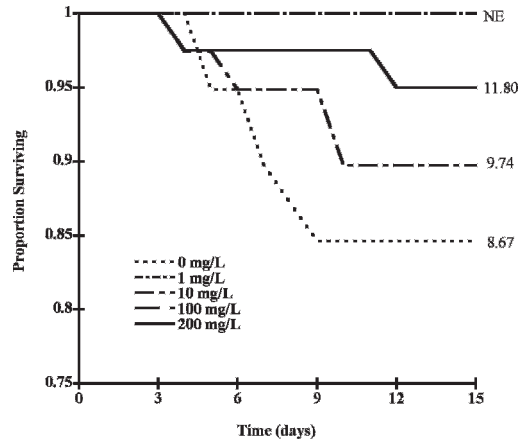

FIG. 3. Tadpole survival under different concentrations of phosphate through time. Numbers to the right of the lines are the Kaplan-Meier estimates of the average survival time in days. NE = not estimable because no deaths were recorded in that treatment. Note that the 1-mg/L and 100-mg/L survival curves are coincident and that Kaplan-Meier survival times are underestimates caused by the few number of deaths recorded.

all other treatments. However, the Tukey comparison identified no significantly different pairs of treatments in SVL. The effect was only marginally significant in body condition. Additionally, control tadpoles were significantly smaller in mass ($F_{4,155} = 2.70$, $P = 0.03$; Fig. 4A) than the 100-mg/L treatment. There were no differences in mass, SVL, or body condition between fungus levels ($F_{1,155} \leq 0.16$, $P \geq 0.69$).

For FA analyses, measurement error was low for both eye width ($F_{185,744} = 3.38$, $P < 0.0001$) and the distance from eye to nare ($F_{185,744} = 2.04$, $P < 0.0001$). For the distance from eye to nare, there was no directional asymmetry ($F_{185,185} = 2.28$, $P = 0.13$) or size dependence ($F_{1,184} = 0.01$, $P = 0.92$) present. There was no difference among treatments ($F_{4,155} = 0.35$, $P = 0.85$) or fungus level ($F_{1,155} = 0.91$, $P = 0.34$) in FA of the distance from eye to nare. Directional asymmetry was present for eye width ($F_{185,185} = 3.95$, $P = 0.05$) with a larger right side on average. The degree of directional asymmetry did not differ among treatments ($F_{4,155} = 0.52$, $P = 0.72$) or fungus level ($F_{1,155} = 0.00$, $P = 0.98$), and the directionality was corrected for by subtracting -1.04 from all data points for further analysis. There was no size dependence present ($F_{1,184} = 0.02$, $P = 0.88$). After correction for directional asymmetry, there were no differences in FA of eye width among treatments ($F_{4,155} = 1.71$, $P = 0.15$), but fungus level 1 had higher FA than fungus level 2 ($F_{1,155} = 4.99$, $P = 0.03$).

DISCUSSION

Phosphate appears to have little effect on *H. chrysoscelis* tadpoles during the 15-day period of this study. There was no significant trend in survival, growth, or developmental stability in relation to increasing concentrations of phosphate. However, it is possible that the presence of the fungus *Saprolegnia* obscured some effects, especially because of the higher amounts of fungus in the control and lower amounts in the 200-mg/L PO_4-P treatment. Although fungal level was not a significant effect (except for eye width FA), the fungus assessment was only performed at the end of the study and may provide limited insight into the tadpole's fungus exposure. The relationship between fungus and phosphate concentration is somewhat paradoxical, considering that phosphate is known to stimulate growth and reproduction of *Saprolegnia ferax* (Pieters, 1915) and *Saprolegnia parasitica* (Kanouse, 1932), two common species parasitic on amphibians, in laboratory conditions (Whitaker and Wright, 2001). Also, it is

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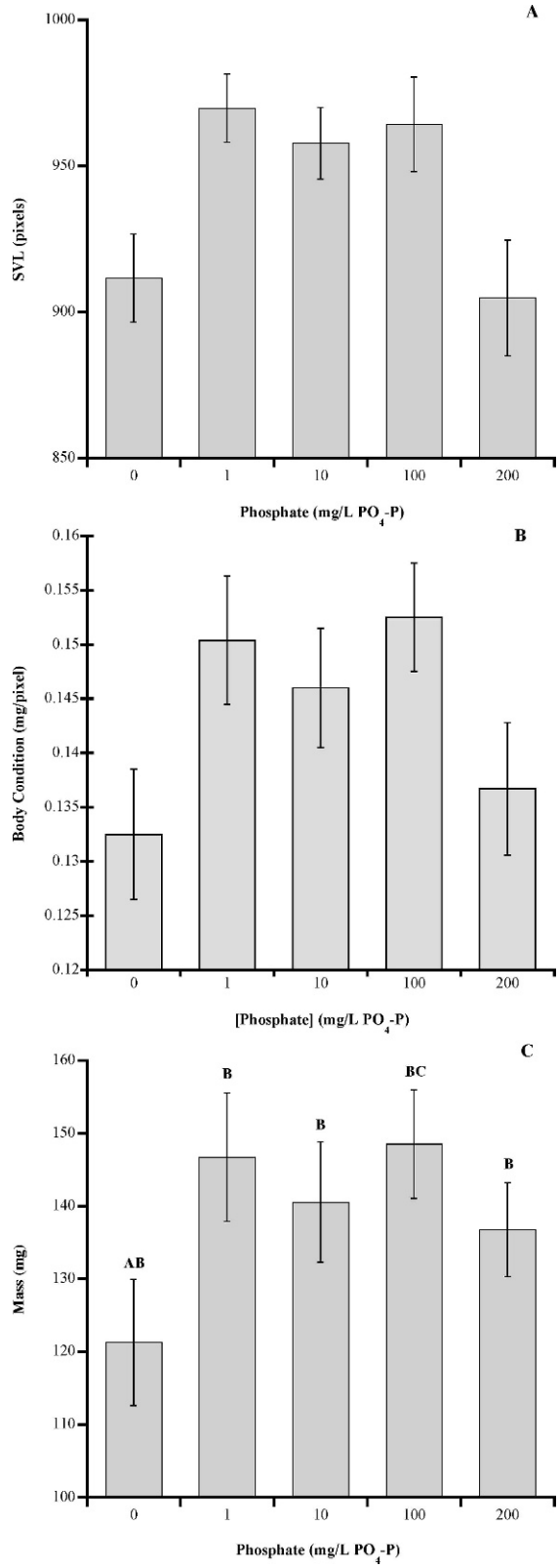
FIG. 4. Least-square mean (±SE) tadpole SVL (A), body condition (B), and mass (C) by phosphate concentration after a 15-day exposure. Groups with different letters are significantly different in mass (C) using the Tukey pairwise comparison. There were no significant differences in SVL (A) or body condition (B).


A
SVL (pixels)
1000
950
900
850
0
1
10
100
200
Phosphate (mg/L PO4-P)
B
Body Condition (mg/pixel)
0.16
0.155
0.15
0.145
0.14
0.135
0.13
0.125
0.12
0
1
10
100
200
[Phosphate] (mg/L PO4-P)
C
Mass (mg)
160
150
140
130
120
110
100
AB
B
B
BC
B
0
1
10
100
200
Phosphate (mg/L PO4-P)

unlikely that a high pH inhibited the growth of *Saprolegnia*, because *S. parasitica* and *S. ferax* grow well in waters with pH ranging from 7–10 (Khulbe, 1980; Koeypudsa et al., 2005).

There were few differences in the size and body condition of the tadpoles among treatments. A general, although nonsignificant, trend was found showing the control and 200-mg/L treatment to have shorter body length and lower body condition than the intermediate treatments. This trend may be an indication that the 200-mg/L treatment had sublethal effects on the tadpoles, which may have been more readily evident if the study duration was longer. However, even if 200 mg/L PO_4-P does negatively affect *H. chrysoscelis* tadpoles, the concentration is so high that it is unlikely to ever occur in the environment (Correll, 1998). Additionally, the control tadpoles were smaller in mass than the tadpoles from the other treatments. It is possible that phosphate is advantageous for this species, although this is not definitive. Elevated phosphate concentrations were found to increase survival in comparison to controls in *Limnodynastes peronii*, but a concentration of 15 mg/L PO_4-P was found to decrease survival in *Litoria aurea* (Hamer et al., 2004). There may be underlying physiological differences between these species that account for differences in response to elevated levels of phosphate (Smith, 2007).

Although phosphate may not be directly toxic to amphibians at any realistic concentration, phosphate may be indirectly toxic. Increases in phosphate concentration raised the pH of the test water substantially. A higher pH can have direct and indirect effects on tadpole performance (Punzo, 1983; Padhye and Ghate, 1988; Fiorimanti et al., 1997), but increased pH did not appear to affect tadpoles in this study. However, pH-induced toxicity could also occur through the increased toxicity of other chemicals. Toxicity of the herbicide Vision® is greatly enhanced with a pH of 7.5, when compared to 5.5, at levels found in impacted waters (Chen et al., 2004; Edginton et al., 2004). Additional experiments should explore these possible synergistic effects.

In natural populations, increased phosphate concentrations could alter food availability dependent on whether phosphate is a limiting nutrient for algae. When phosphate is limiting, increases in phosphate would increase algal biomass (Pitcairn and Hawkes, 1973), which should have a positive effect on tadpoles through increased food availability. However, when phosphate is not limiting, phosphate additions could decrease tadpole food resources through increasing pH. Relyea (2006) found that a pH of 8 lowered growth rates in *Rana clamitans* and *Rana catesbeiana* through an associated decrease in periphyton biomass.

Clearly, more studies need to be conducted on the impacts of phosphate on amphibian populations and ecosystems as a whole. More research is needed on the direct toxicity of phosphate and the physiological differences among species with different responses to elevated phosphate concentrations (Hamer et al., 2004; Smith, 2007; this study), including longer studies with endpoints at metamorphosis (e.g., Hamer et al., 2004). Additionally, because phosphate is likely to occur in conjunction with other chemicals, the synergistic effects of phosphate and other chemicals commonly applied to agricultural fields and present in wastewater need to be evaluated. Chemicals such as phosphate also need to be examined in a community context, so that trophic interactions and indirect effects can be determined (Boone and James, 2005; Boone and Bridges-Britton, 2006).

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