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EFFECTS OF PULSED NITRATE EXPOSURE ON AMPHIBIAN DEVELOPMENT

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Abstract—Most toxicity tests investigate constant concentrations of a chemical. Concentrations of many compounds in the environment are dynamic, however, and individuals may be more sensitive to pulses of a chemical initiated at specific points during development. Realistic toxicity tests are important to accurately assess the toxicity of a compound. *Bufo americanus* and *Hyla chrysoscelis* tadpoles were exposed to concentrations of nitrate, a pervasive stressor in the environment, up to 5 mg/L of NO_3–N in constant concentrations and in pulses at three different points during development. At the termination of the experiments, individuals were measured for developmental stability (DS) and traditional fitness correlates (size, body condition, and time to metamorphosis). No significant differences were found in these measures between treatments and controls in *B. americanus* tadpoles. In *H. chrysoscelis*, however, more extreme directional asymmetry was found in the middle and late pulses, indicating that the sudden change in concentration of nitrate decreased the level of DS in tadpoles. These results indicate that nitrate has subtle but important effects at low doses, and they suggest that species may be better able to deal with pulses that occur early rather than late in development. A greater understanding about the effects of pulses will help conservation biologists to manage populations and prevent population declines.

Keywords—Developmental stability Ecotoxicology *Hyla* *Bufo* Pulsed exposure

INTRODUCTION

Conservation biologists are concerned with the persistence of populations through time, which is directly related to a population's ability to deal with stress [1]. To properly manage populations and prevent declines, it is important for scientists to understand how different types of stresses and their timing affect populations. Some stresses are initiated gradually and occur over long periods of time; others are relatively sudden and short term. This second type of stress is more dynamic, and its effects are more difficult to predict. Toxicants in the environment exhibit both temporal regimes. Some chemicals are persistent, whereas others, such as nutrients, are very dynamic, exhibiting changes in concentration on various time scales [2]. One example is the natural influx (or pulse) of nutrients, such as nitrate, as a result of runoff during a storm event [3].

Most toxicology studies examine the effects of constant concentrations of various chemical compounds [4], but pulses may affect organisms differently than static levels do. Additionally, organisms may be more sensitive to pulses of toxicants at different points in their development. This could be especially important for amphibians because of the vast morphological changes that occur during metamorphosis [5]. There may be specific time periods, such as just after hatching or during metamorphosis, when hypersensitivity to toxicants or other environmental stresses occurs. Previous studies have examined the effects of pulses, including various insecticides [6,7], but to our knowledge, no previous study has compared pulses at different points during development.

Concentrations of nutrients, such as nitrogen, are dynamic in aquatic systems. Human influence has greatly altered the global nitrogen cycle over the past 150 years by mobilizing previously unreactive forms of nitrogen [8]. Reactive nitrogen can enter bodies of water through fertilizer runoff from agricultural fields, inputs from livestock, and atmospheric deposition [9,10]. Common forms of reactive nitrogen include ammonium, nitrite, and nitrate, but because of nitrification, ammonium and nitrite are converted to nitrate in the presence of oxygen [2]. For this reason, nitrates are the most concentrated form of inorganic nitrogen in aquatic systems [2] and should be a focus when examining the effects of increased nitrogen in the environment. One important effect of nitrates is toxicity to aquatic organisms, such as amphibians [11]. Although less toxic to amphibians compared with ammonium or nitrite [12], nitrates still have lethal effects at relatively low concentrations [13]. Many nitrate toxicity tests have examined lethal impacts of nitrate [13], but relatively few studies have focused on sublethal effects (C.S. Meredith and H.H. Whiteman, unpublished data).

One way to assess the impacts of toxicants, such as nitrate, is through developmental stability (DS)—that is, the ability to buffer stresses during development [14]. The most widely used measure for DS is fluctuating asymmetry (FA), which examines the frequencies within a population of normally distributed, minor deviations from perfect bilateral symmetry with a mean of zero [14,15]. Scientists can use FA to assess the impacts of various environmental factors through the physical manifestation of asymmetrical development, and FA has been proposed as an early warning system to detect stress in a population before that stress has negative consequences for fitness [16,17]. Another, more controversial measure of DS is directional asymmetry (DA), which is similar to FA except that the mean is not zero [14]. Recently, studies have shown a shift from FA to DA with extreme levels of stress [18,19].

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The knowledge about stress gained from FA and DA could help managers to better identify vulnerable populations and prevent population declines [17], which is especially important for amphibian populations in light of recent global declines [20,21].

The present study compares the effects of sublethal nitrate concentrations at constant levels and pulses at different points in the larval period of anurans. Two species, *Bufo americanus* and *Hyla chrysoscelis*, were exposed in laboratory conditions to low levels of nitrate (up to 5 mg/L of NO_3–N) at constant concentrations through the larval period and as pulses timed for early, middle, and late in the larval period. At the termination of each experiment, tadpoles were assessed for traditional measures of fitness (size, time to metamorphosis, and body condition) and for DS in various traits through measures of FA and DA. Additionally, the nitrogen content of the tail muscle tissue was measured to assess the bioaccumulation of nitrogen in tadpoles exposed to elevated levels of nitrate.

MATERIALS AND METHODS

Bufo americanus *experiment*

Bufo americanus eggs were collected from two roadside ditches on April 1, 2006, in Trigg County (KY, USA) from six pairs that were still in amplexus at the time of collection. Eggs were allowed to develop at room temperature in the water in which they were collected. Hatching occurred 3 d after collection. The experiment started 9 d after hatching, when all tadpoles were at Gosner stage 24 or 25 [5]. Each tadpole was randomly assigned to one of seven treatments, each with 12 replicates, with each replicate represented by a glass culture dish containing four tadpoles and 100 ml of the treatment solution, which consisted of sodium nitrate (in the appropriate treatments) dissolved in a very soft (pH 6.4–6.8; hardness, 10–13 mg/L as $CaCO_3$), reconstituted freshwater solution [4]. The treatments included four with constant concentrations (0, 1, 2.5, and 5 mg/L of NO_3–N) and three with pulses of 5 mg/L of NO_3–N at different stages of development (early, middle, and late) (Fig. 1A). The early pulse started on day 1 of the experiment; the middle pulse on day 20, when 50% of the individuals in that treatment reached Gosner stage 31 [5]; and the late pulse on day 26, when 50% of the individuals in that treatment reached Gosner stage 36 [5]. These developmental stages were chosen for their easy recognition and for their timing at approximately halfway through the larval period for the middle pulse and approximately one week before metamorphosis for the late pulse. Each pulse consisted of a 6-d period, beginning with 2 d at 5 mg/L of NO_3–N. At each water change, which were performed 2 d apart, the water was replaced with that of a lower concentration until the end of the pulse, when water with 0 mg/L of NO_3–N was placed in the dishes (Fig. 1B). The gradual decrease in nitrate concentration simulated a natural decrease in concentration because of the uptake of nitrate by algae. The concentrations were chosen within the range of anthropogenic influence [2].

The water was changed every 2 to 3 d. During a pulse, the water was always changed every 2 d so that each pulse was identical. During a water change, when a new concentration was added or if a bacterial or fungal film was present, the dish was rinsed thoroughly with distilled, deionized water before the addition of new water. Additionally, during one water change per week, water samples were taken from one randomly chosen dish from each treatment and analyzed using a LACHAT 8000 Quickchem® Flow Injection Analyzer to check nitrate

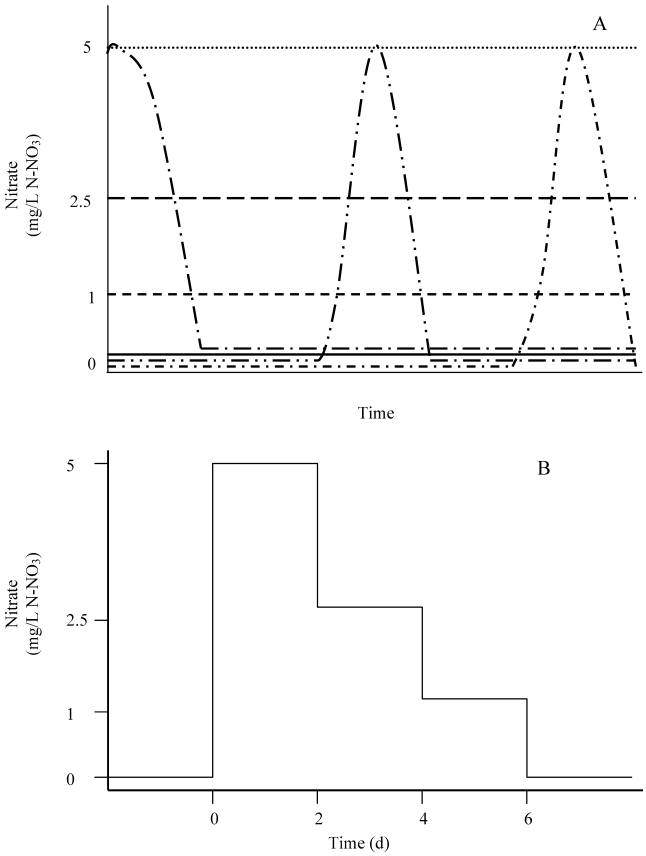

Fig. 1. Concentrations of nitrate through time for each of seven treatments for both *Bufo americanus* and *Hyla chrysoscelis* (**A**). Treatments include four constant concentrations at 0 mg/L (solid line), 1 mg/L (short dashed line), 2.5 mg/L (long-dashed line), and 5 mg/L (dotted line) and an early pulse (long-dash-dot line), middle pulse (dash-dot-dot line), and late pulse (short-dash-dot line). The pulse begins at day 0 and ends at day 6 (**B**). Water changes occur at days 0, 2, 4, and 6.

concentrations. On the days when the water was not replaced, 10 ml of reconstituted freshwater were added to every dish to counteract the concentrating effects of evaporation. The temperature (to the nearest 0.25°C), pH (to the nearest 0.01 unit), and dissolved oxygen (to the nearest 0.01 mg/L) were measured every 1 to 2 d in a randomly chosen dish from each treatment using a mercury thermometer, an Orion pH meter (model 210A), and a Yellow Springs Instruments dissolved oxygen meter (model 54A), respectively. Tadpoles were fed a mixture of ground alfalfa pellets and fish food at a 3:1 ratio ad libitum.

The experiment was terminated on day 32. On the final day, tadpoles were weighed, staged [5], photographed, assessed for deformities, and killed. Mass was recorded to the nearest 0.01 g with an Acculab model V-200 balance. Tadpoles were positioned and photographed three times each to assess measurement error (J.E. Earl and H.H. Whiteman, unpublished data) using a Pixera model PVC 100C through a microscope at a ×40 magnification. All photographs were taken with 1,280 × 1,024 pixels. Photographs were measured for the eye width, the distance from the eye to the nare, and the snout-to-vent length (SVL) using ImageJ software (http://rsb.info.nih.gov/ij).

Hyla chrysoscelis *experiment*

On August 4, 2006, two *H. chrysoscelis* amplecting pairs were captured at Hancock Biological Station in Calloway

County (KY, USA) and allowed to lay eggs under laboratory conditions. The following morning, two additional egg masses were collected from two different artificial ponds at the station. Eggs hatched within 2 d, and the experiment started 3 d after hatching (Gosner stage 24–25), when tadpoles were introduced into 100-ml glass dishes. Tadpoles were randomly assigned to treatments, which were identical to those described for *B. americanus*. In this experiment, however, each replicate was represented by one tadpole per dish, with 40 replicates per treatment, to increase statistical power. Dishes were placed in three incubators, with equal proportions of each treatment in each incubator. Incubators were set to 29°C and a 12:12-h light: dark photoperiod. The early pulse began on day 1 of the experiment; the middle pulse on day 14, when 50% of the individuals reached Gosner stage 32 [5]; and the late pulse on day 22, when 50% of the individuals reached Gosner stage 36 [5]. Water changes, water sampling, and feeding regimens were identical to that for the *B. americanus* study. Incubator thermometers were used to assess the temperature in each incubator on a daily basis. The pH was measured in one randomly chosen dish from each treatment every 2 to 3 d. Dissolved oxygen was not measured because of the constrained space of the incubators.

Each individual was weighed, photographed, and killed at metamorphosis, which was defined as Gosner stage 42 [5]. The experiment ended when all individuals reached metamorphosis. Weight and photograph protocols were as described above, except that one set of photographs was taken of the body and one set was taken of the legs for greater precision. Photographs were measured for the eye width, the distance from eye to nare, the radio-ulnal length, the tibiofibulal length, the calcaneum length, and the SVL using ImageJ software.

Statistical analyses

All statistical analyses were performed using SAS/STAT® (SAS Institute) [22]. Survival analysis for the *H. chrysoscelis* experiment was performed using the Kaplan–Meier test using incubator as a blocking variable and all possible pair combinations of treatments with the Bonferroni correction adjusting the α value to 0.002. All other tests were conducted using $\alpha = 0.05$. Because of the presence of four tadpoles in each replicate in the *B. americanus* experiment, the Kaplan–Meier test could not be used. Instead, a repeated-measures analysis of variance (ANOVA) was performed on the percentage survival for each dish for each day of the experiment. One-way ANOVAs were performed on all physicochemical data to determine differences among treatments and on developmental stage and time to metamorphosis to determine the effects of treatments. Before analysis, SVL was assessed for measurement error using a one-way ANOVA. If measurement error was not significant, the three measurements of SVL were averaged. Body condition was calculated by dividing mass by SVL. For the *B. americanus* experiment, a dish average was taken and used for all analyses, and for the *H. chrysoscelis* experiment, incubator was used as a blocking variable. For mass, SVL, and body condition, a multivariable regression was performed with Gosner stage (*B. americanus*) or time to metamorphosis (*H. chrysoscelis*) and dummy variables to represent the treatments and incubators (*H. chrysoscelis*).

To evaluate DS, all traits were first tested for measurement error, DA, and size dependence. Measurement error was assessed with a two-way, mixed-model ANOVA using individual as a random variable and side (left or right) as a fixed variable. The significance of this interaction indicated that the between-sides variation was greater than the measurement error; thus, measurement error was low enough for further analysis [15]. This analysis also assessed DA. The three measurements were then averaged for each side. The left side was subtracted from the right side for each individual for each trait to produce an asymmetry index [15]. A simple linear regression was performed on the FA index by the average size of the trait to determine size dependence of FA [15]. For traits with low measurement error, no DA, and no size dependence, the absolute value of the asymmetry index was taken and used in a one-way ANOVA to determine differences among treatments. For traits with significant DA, an ANOVA on the signed asymmetry was performed to determine whether the degree of DA varied among treatments. Additionally, the distribution was centered by subtracting the mean asymmetry from all points to correct for DA, creating a new asymmetry index [15]. The absolute value was taken of the asymmetry index and analyzed using an ANOVA to assess differences among treatments in FA. 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 0.0113.

RESULTS

Bufo americanus *experiment*

Measured nitrate concentrations from water samples generally were very close to target levels. The target levels of 1 mg/L of NO_3–N ranged from 0.86 to 1.00 mg/L, the target levels of 2.5 mg/L ranged from 1.85 to 2.57 mg/L, and the target levels of 5 mg/L ranged from 4.14 to 5.25 mg/L. No evidence of any contamination was found; control levels never exceeded 0.18 mg/L and usually were below detection limits. Severe dilution was found across all treatments compared to control levels on May 6 and May 14, likely because of the presence of obvious bacterial films. No difference was found in temperature ($p = 0.99$; mean $\pm$ standard error, 21.79 $\pm$ 0.11°C), dissolved oxygen ($p = 0.48$; 6.73 $\pm$ 0.22 mg/L), or pH ($p = 0.82$; 7.33 $\pm$ 0.02) among treatments.

No difference was found in survival among treatments overall ($p = 0.89$) or in interaction with time ($p = 1.00$). Some tadpoles had deformities in the form of spinal curvatures, but no difference was found among treatments in the percentage of malformed tadpoles by dish ($p = 0.95$). Gosner stage of individuals at experiment termination did not vary among treatments ($p = 0.79$), and SVL did not exhibit significant measurement error ($p < 0.0001$). Differences among treatments were found in mass (Fig. 2A), SVL (Fig. 2B), and body condition (Fig. 2C). In all three models, Gosner stage was not significant as an overall covariate but was significant in interaction with some dummy variables, indicating a significant relationship between Gosner stage and the response variable in some treatments. In all regression models, the late pulse and the constant 2.5 mg/L treatments produced significant relationships between the response variable and Gosner stage (all $p < 0.008$). For SVL, a significant relationship with Gosner stage was found in the constant 1 mg/L and the middle pulse treatments (all $p < 0.012$) (Fig. 2B).

Low measurement error occurred in eye width ($p < 0.0001$) and in the distance from eye to nare ($p < 0.0001$). Both traits exhibited significant DA (eye width, $p < 0.0001$; distance from eye to nare, $p < 0.0001$). No difference was found among treatments in DA of either trait (eye width, $p = 0.61$; distance

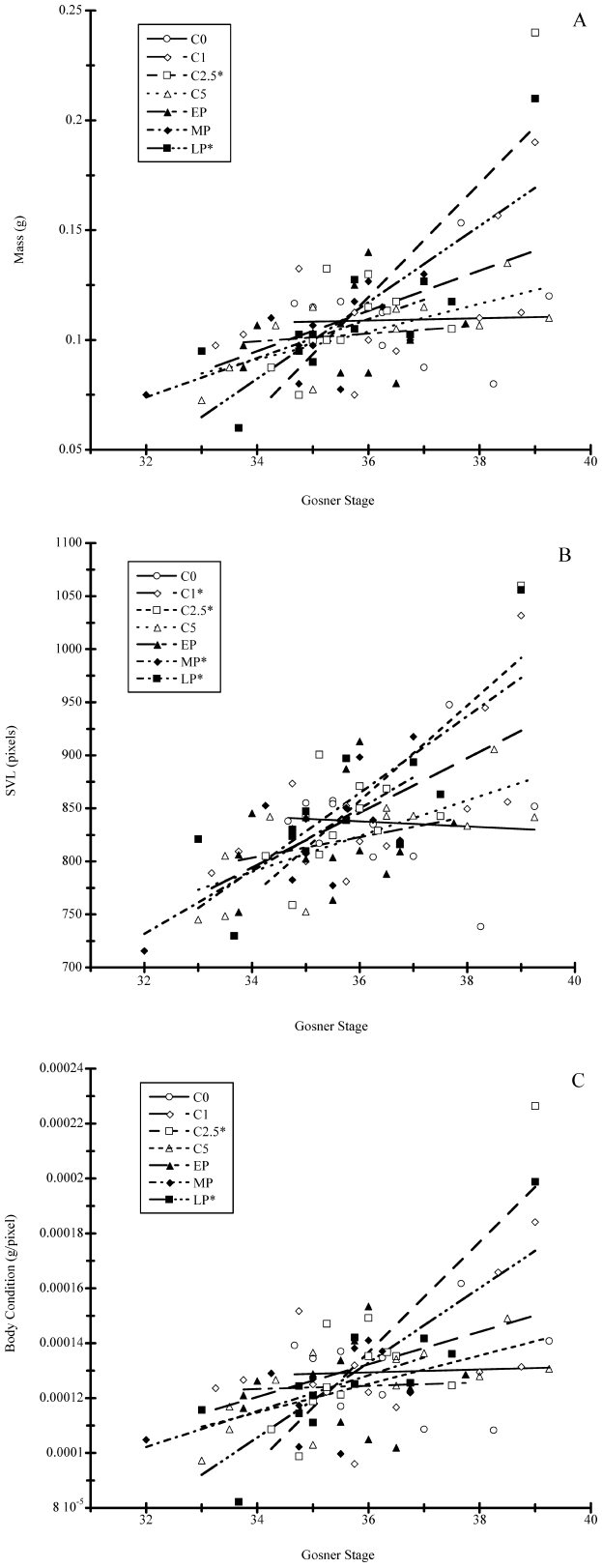

Fig. 2. Relationship between Gosner stage and mass (**A**), snout-to-vent length (SVL) (**B**), and body condition (**C**) by treatment in *Bufo americanus*. Asterisks indicate that the slope and intercept are significantly different from zero. Treatments without an asterisk do not have a significant relationship between mass and Gosner stage. C0 = constant, 0 mg/L of NO_3–N; C1 = constant, 1 mg/L of NO_3–N; C2.5 = constant, 2.5 mg/L of NO_3–N; C5 = constant, 5 mg/L of NO_3–N; EP = early pulse; MP = middle pulse; LP = late pulse.

from eye to nare, $p = 0.88$). Eye width was centered by subtracting −0.28 from each measurement after taking dish averages, and the distance from eye to nare was centered by subtracting 2.22 from each measurement. No difference was found among treatments in FA in eye width ($p = 0.24$) or in the distance from eye to nare ($p = 0.15$) after centering.

Hyla chrysoscelis *experiment*

Measured nitrate concentrations generally were lower than target levels. Target levels of 1 mg/L of NO_3–N ranged from 0.70 to 0.80 mg/L, target levels of 2.5 mg/L ranged from 1.31 to 2.13 mg/L, and target levels of 5 mg/L ranged from 3.53 to 4.40 mg/L. No evidence was found of contamination at any point; the controls never had concentrations of greater than 0.17 mg/L. Severe dilution almost to control levels across treatments was evident on August 25 and September 3, possibly because of uptake from persistent bacterial films present on the dishes throughout the experiment. Solutions tested before use in dishes were always within 5% of target levels. The pH did not differ significantly among treatments ($p = 0.57$; 7.09 ± 0.04), and temperature did not differ significantly among incubators ($p = 0.12$; 28.90 ± 0.09°C).

No significant difference in survival was found among treatments in incubators 1 and 2 (all $p > 0.46$), but in incubator 3, a significant difference was found ($p = 0.004$). Significant differences occurred between the middle pulse and the constant 5 and 2.5 mg/L treatments, which showed reduced survival. A significant effect of incubator ($p < 0.0001$) and of the interaction between treatment and incubator ($p = 0.001$) in time to metamorphosis was found, but no treatment was significantly different from the control. Time to metamorphosis, treatment, and incubator were not significant predictors of SVL (all $p > 0.11$), which had low measurement error ($p < 0.0001$). Incubator was a significant predictor of body condition ($p = 0.04$), however, and a marginally significant positive relationship was observed between time to metamorphosis and body condition ($p = 0.05$). Differences in mass among treatments were found using time to metamorphosis as a covariate (Fig. 3), but only the late pulse showed a significant relationship between mass and time to metamorphosis ($p = 0.01$).

All traits used in DS analysis, except the distance from eye to nare, had low measurement error (Table 1); the distance from eye to nare was excluded from further analysis. Only eye width did not have significant DA (Table 1). The treatments differed significantly in DA of radio-ulnal length ($p = 0.005$) (Fig. 4A) and calcaneum length ($p = 0.01$) (Fig. 4B). No effect of incubator (all $p > 0.47$) or of the interaction between treatment and incubator (all $p > 0.13$) was found in either trait. No difference was found among treatments or incubators in DA of tibio-fibulal length (all $p > 0.07$), which also displayed significant size dependence (Table 1). To correct for this, the natural log was taken of both sides, and a new FA index was formed by subtracting the natural log of the left side from the natural log of the right side, which eliminated the size dependence ($p = 0.08$). Directional asymmetry was still evident, and 10.93 was subtracted from each measurement to center the distribution. Analysis of the centered data revealed no differences among treatments in any trait (all $p > 0.10$).

DISCUSSION

The main goal of the present study was to compare the effects of nitrate in constant concentrations and in pulses, par-

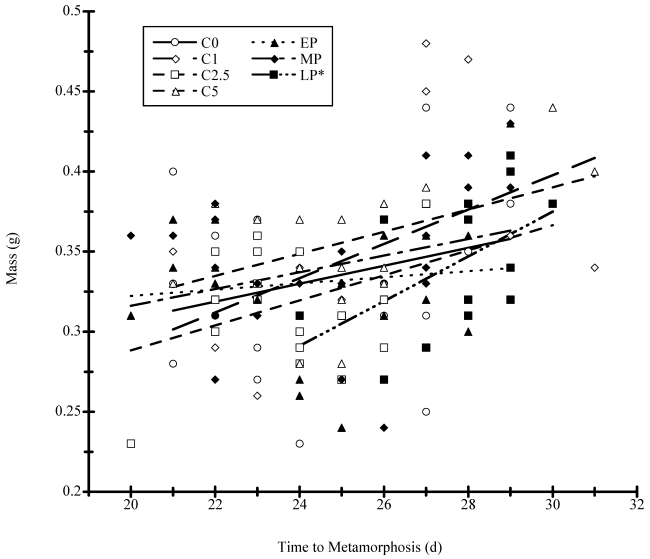

Fig. 3. Differences among treatments in the relationship between mass and time to metamorphosis in *Hyla chrysoscelis*. Asterisk indicates the treatment with a slope significantly different from zero. C0 = constant, 0 mg/L of NO_3–N; C1 = constant, 1 mg/L of NO_3–N; C2.5 = constant, 2.5 mg/L of NO_3–N; C5 = constant, 5 mg/L of NO_3–N; EP = early pulse; MP = middle pulse; LP = late pulse.

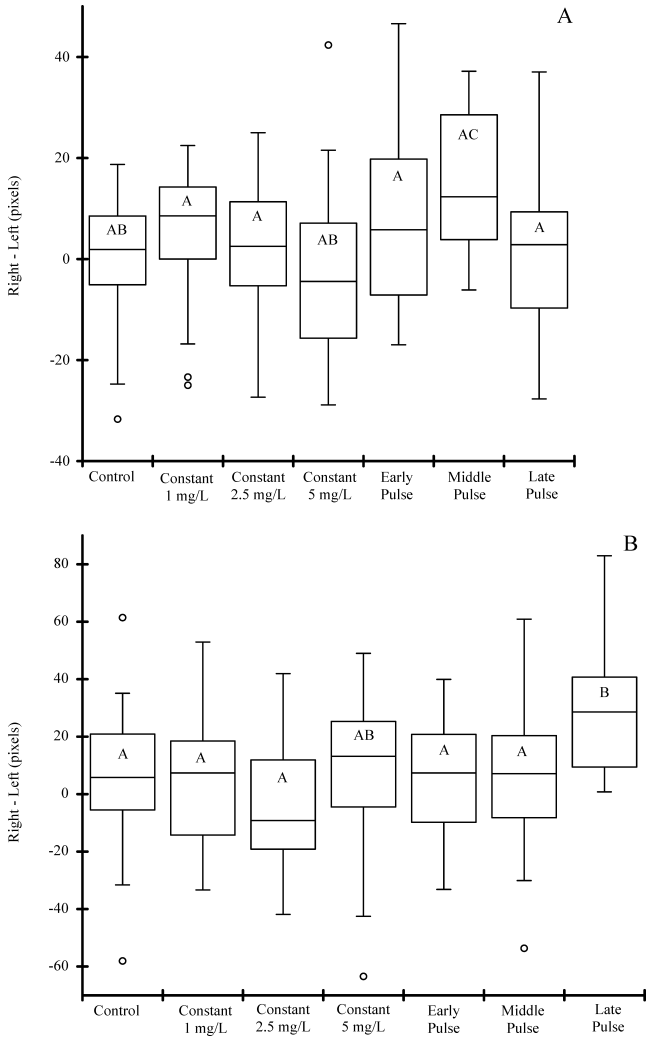

Fig. 4. Differences in directional asymmetry in radio-ulnal length (**A**) and calcaneum length (**B**) for *Hyla chrysoscelis* tadpoles. Distributions for each treatment are presented as box-plots. Treatments with different uppercase letters are significantly different using Tukey's pairwise comparisons.

ticularly in regard to the DS of tadpoles and metamorphs. Nitrate treatments increased DA in two traits in *H. chrysoscelis* metamorphs. The middle pulse treatment had significantly greater DA in radio-ulnal length than controls, and the late pulse had greater DA in calcaneum length than all treatments except the constant 5 mg/L of NO_3–N. This trend was not found in the *B. americanus* experiment, perhaps because of the termination of that experiment before metamorphosis, resulting in no limb measurements. It also is possible that the concentrations of nitrate used in this experiment were simply not high enough to induce lowered levels of DS in *B. americanus*.

Although many studies have reported DA (see, e.g., [23,24]), few studies have looked for correlations between DA and environmental stress, but several have shown that toxicants can induce high levels of FA (e.g., [25,26]). Some researchers advise that all traits with DA be eliminated from DS analysis [15], arguing that the heritability of DA is unclear [27] (though the heritability of FA also is unclear [14]) and that the ideal state cannot be known [27]. The more extreme DA found in the late pulse treatment, however, suggests that traits with DA should not simply be discarded in studies of DS [28]. Also, recent studies have shown that extreme levels of stress, such as the effects of habitat disturbance on birds [18] and high levels of lead and benzene on fruit flies [19], induce DA [29,30].

The present study is consistent with the hypothesis that extreme stress can induce DA: Pulse treatments led to higher DA, perhaps because sudden changes in concentration are much more stressful than constant concentrations. It also may be true, however, that pulses disrupt traits that are rapidly developing. The late pulse was initiated at Gosner stage 36, which is during calcaneum development [5]. Forelimb development generally is one stage behind that of hindlimb development [31], so the radio-ulna would have been developing during the initiation of the middle pulse. Thus, the pulses appear to have lowered DS, manifested as DA. It is possible that the early pulse caused a similar developmental disruption,

Table 1. Statistics for testing the assumptions of fluctuating asymmetry analysis for *Hyla chrysoscelis*[a]

Trait	Measurement error	DA[a]	DA correction[b] (pixels)	Size dependence
Eye width	2.55 (<0.0001)[a]	0.19 (0.66)[a]	NA	1.07 (0.30)
Radio-ulnal length	1.27 (0.03)	11.78 (0.0008)	−4.22	2.27 (0.13)
Tibio-fibulal length	2.10 (<0.0001)	308.33 (<0.0001)	−17.79	10.93 (0.0012)
Calcaneum length	1.37 (0.006)	6.22 (0.01)	−5.49	2.35 (0.13)

[a] *F*-statistics are reported with *p* values in parentheses. DA = directional asymmetry.
[b] DA correction refers to values added to each measurement to center the distribution.

but that the tadpoles had time to compensate for it during the remainder of the larval period. To detect such a trend, measurements would have to be taken throughout the experiment. Similar compensation hypotheses have been suggested for the lack of high FA in damselflies exposed to pulses of the insecticide carbaryl in mesocosms [32]. Further research is needed to assess whether compensation can correct for the DA evident in the middle and late pulse treatments and how much time or growth is required for that compensation. If individuals can compensate for asymmetrical development, the length of time required may vary based on the extremity of DA—and, therefore, the amount of DS—evident in the population. If compensation occurs relatively quickly, this would indicate that the developmental stress is low or that these traits are highly plastic.

Alternatively, while compensational development may have taken place in individuals from the early pulse, the tadpoles also may be less sensitive to pulses early in development. Metamorphosis is a sensitive period of development [31], and the increased DA in the middle and late pulse treatments may be an indicator of this. Thus, for explosive breeders like *B. americanus* [33] and *Scaphiopus holbrooki* [34], it may be prudent to minimize application of fertilizer during the time of year when most individuals are going through metamorphosis. For species with a more prolonged breeding period, such as *H. chrysoscelis* [35], it is more difficult to make recommendations for the timing of fertilizer application, but application may need to be restricted during the peak of metamorph emergence, especially in vulnerable populations that frequently breed in ponds adjacent to agricultural areas.

Additionally, to compare constant concentrations of nitrate to pulses, traditional fitness correlates were used along with DS. Few consistent differences were found among the treatments. It appears that concentrations of nitrate up to 5 mg/L are benign to *H. chrysoscelis* and *B. americanus* tadpoles, because no significant differences were found among treatments and controls in traditional measures of fitness. In the *H. chrysoscelis* experiment, survival was significantly reduced in the constant 2.5 and 5 mg/L in comparison with the middle pulse in one incubator, but no treatment was significantly different from the control. Meredith and Whiteman (unpublished data) also found no difference between the control and 5 mg/L treatment in *H. chrysoscelis* tadpoles. Individuals from the late pulse had a longer larval period compared with individuals from the constant 1 and 2.5 mg/L treatments but, again, were not significantly different from controls.

Other differences occurred among treatments in the relationship between size and time to metamorphosis (in *H. chrysoscelis*) or developmental stage (in *B. americanus*). A significant positive relationship between size and time to metamorphosis or developmental stage was found in both species for the late pulse, and no relationship was found for the controls. A similar trend was found in a study of the fungicide triphenyltin: The control and lower concentrations had a negative relationship between size and time to metamorphosis, but the highest concentration had a positive relationship [36]. Metamorphosing early and at a larger size is thought to increase fitness [37], so when size and time to metamorphosis have a negative relationship or no relationship, individuals can have the fitness benefits of both an earlier and larger metamorphosis. When a positive relationship exists between size and time to metamorphosis, however, individuals can either metamorphose early or at a large size (i.e., a fitness trade-off), which may be a general response to growth-limiting, environmental stress [36].

The levels of nitrate used in the present study clearly affected tadpole growth and development, but the concentrations used may have greater influence in conjunction with other stressors, such as high competition, the presence of predators, or other anthropogenic pollutants, such as herbicides. Larger-scale mesocosm experiments are needed to assess the affects of high nitrate concentrations on tadpoles in a community framework [38]. It also may be important to investigate the effects of nitrate in the context of algal nutrient availability, particularly the availability of phosphate and silica along with nitrate. Some nutrient ratios may stimulate the growth of algae [2], an important food source for tadpoles [32], whereas others may provide an excess of nitrate [2], which may lower the fitness of tadpoles.

Conservation biologists are concerned with the ability of populations to deal with stress and to persist through evolutionary time scales [1]. The present study shows the importance of short-term pulses of a toxicant and the developmental timing of those pulses. Many stresses that affect populations occur in relatively short-term pulses. Populations may be better able to cope with gradual, long-term disturbances in comparison with sudden, short-term stresses, particularly those of anthropogenic origin. It is important for conservation biologists to understand the effects of these short-term pulses, the degree to which the response to a pulse varies with developmental timing, and the persistence of the effects from a pulse. Ultimately, this type of information will help biologists to manage populations and prevent population declines.

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