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JUVENILE AMPHIBIANS DO NOT AVOID POTENTIALLY LETHAL LEVELS OF UREA ON SOIL SUBSTRATE

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Abstract—We examined the effects of a forest fertilizer (urea) on newly metamorphosed terrestrial amphibians (Western toads, *Bufo boreas;* Cascades frogs, *Rana cascadae*; long-toed salamanders, *Ambystoma macrodactylum*; and roughskin newts, *Taricha granulosa*). We examined avoidance behavior of Western toads and Cascades frogs on both paper towel and soil substrates dosed with urea (control and 100 kg N/ha and an additional treatment of 50 kg N/ha for Western toads on soil substrate) and avoidance behavior of long-toed salamanders on soil substrate dosed with urea. We further examined the survival and feeding behavior of all four species exposed to urea on soil substrate (100 kg N/ha) for 5 d. Juvenile Western toads and Cascades frogs avoided paper towels dosed with urea but did not avoid urea-dosed soil substrate. However, Western toads and Cascades frogs both suffered significant mortality when exposed to urea on a soil substrate for 5 d. Furthermore, after adjusting for weight, we found that urea-exposed juvenile Western toads and Cascades frogs consumed significantly fewer prey items (crickets) compared with nonexposed control animals. Long-toed salamanders did not discriminate against soil substrate dosed with urea, and neither long-toed salamanders nor roughskin newts died or reduced prey consumption as a result of urea exposure. Juvenile amphibians may not be able to detect and avoid harmful levels of urea fertilizer on a natural substrate. Furthermore, anthropogenic stressors such as urea fertilizer can significantly reduce the survival and prey consumption of juvenile amphibians. These effects are important to consider in light of possible threats to the conservation status of many amphibian species.

Keywords—Juvenile amphibian Feeding behavior Avoidance behavior Urea fertilizer

INTRODUCTION

Anthropogenic alteration of the nitrogen cycle is one of the most potentially harmful global environmental problems [1,2]. Anthropogenic nitrogen fixation has dramatically increased in the past 50 years and now exceeds input from all other sources [2]. Indeed, the use of nitrogenous fertilizers has increased by about sevenfold over the past 35 years [1]. These changes in the nitrogen cycle may contribute to global environmental change by altering the ability of ecosystems to respond to increased carbon dioxide, reducing plant species richness [2] and causing eutrophication of aquatic systems [1].

With the increasing demand on the nitrogen cycle, the potential for impacts on wildlife, such as amphibians, also increases. Agricultural practices and forest fertilizer application are both sources of nitrogenous input into amphibian habitats. In agricultural areas, fertilizer runoff can impact aquatic amphibian breeding sites with detrimental consequences to developing amphibians [3–7]. Because amphibians are integral components of many ecosystems [8], the detrimental impact of fertilizer runoff pollution on amphibian populations may affect populations of other organisms. Moreover, amphibians are ideal indicators of environmental stress [8].

Nitrogen pollution in aquatic systems has been associated with lethal and sublethal effects on developing amphibians [5]. For example, exposure of larval *Ambystoma gracile* and *Rana pretiosa* to nitrate (e.g., 12–25 mg/L) caused significant mortality in laboratory tests [3]. Low levels (e.g., less than 2 mg/L) of nitrite, a breakdown product of nitrate, also caused mortality in larvae of a number of amphibian species in the Pacific Northwest, USA (*Rana pretiosa, Bufo boreas, Hyla regilla,* and *Ambystoma gracile*) [3]. Furthermore, low levels (3.5 mg/L) of nitrite delayed metamorphosis and altered behavior of larval Cascades frogs [4]. In another study, larval stages of *Bufo americanus, Pseudacris triseriata, Rana pipiens,* and *Rana clamitans* suffered significant mortality when exposed to nitrate levels similar to those measured in ponds near agricultural areas [6]. Longer term exposures (100 d) revealed reduced feeding, activity, and growth in larvae as a result of nitrate exposure [6]. Similarly, Watt and Oldham [7] found reduced growth of newt larvae (*Triturus vulgaris*) exposed to ammonium nitrate at nitrate concentrations that were elevated compared with what might typically be found in nature (200–500 mg nitrate/L). Nitrate also may interact with other stressors such as low pH and ultraviolet (UV) radiation, reducing survival and activity of larval amphibians [9].

Urea is applied as a forest fertilizer and may be an important route of nitrogen exposure for amphibians inhabiting forest ecosystems. Forest fertilizers may contaminate aquatic systems as runoff, but they may also affect terrestrial-stage amphibians via dermal exposure. In the Pacific Northwest, urea is applied to new forest stands approximately once every eight years (B. Porter, U.S. Forest Service, personal communication). Previous studies have demonstrated that terrestrial salamanders such as *Plethodon vehiculum, Rhyacotriton variegatus,* and *Taricha granulosa* avoid urea at a concentration of 225 kg N/ha [10]. In a 4-d experiment, *P. vehiculum* and *R. variegatus* exhibited mortality at 450 kg urea/ha after a 24-h exposure [10]. Oldham et al. [11] found that ammonium nitrate fertilizer was toxic to adult common frogs (*Rana temporaria*), but these investigators also noted that this fertilizer dissolved rapidly in the field, reducing the likelihood of exposure.

In the current study, we assessed effects of urea on four

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species of recently metamorphosed juvenile amphibians, including Western toads (*Bufo boreas*), Cascades frogs (*Rana cascadae*), long-toed salamanders (*Ambystoma macrodactylum*), and roughskin newts (*Taricha granulosa*). We tested whether Western toads, Cascades frogs, and long-toed salamanders would discriminate between urea-contaminated substrate or noncontaminated substrate (paper towel and soil in the case of Western toads and Cascades frogs, soil only in the case of long-toed salamanders). We also examined the effects of a 5-d urea exposure on the survival and prey consumption of juveniles by all four species.

NATURAL HISTORY AND CONSERVATION STATUS OF STUDY ORGANISMS

Western toads (*B. boreas boreas*) occur throughout the Pacific Northwest from near sea level to high montane habitats [12]. Adult Western toads are mostly terrestrial and nocturnal. Although they may travel large distances at night, adults typically burrow in loose soil during the day [12]. During spring breeding, adults typically congregate at permanent ponds for communal mating. Newly metamorphosed juveniles tend to be highly aggregated [13]. Many populations of Western toads have declined significantly or disappeared completely in parts of their Western range. For example, populations have declined or disappeared from the West Elk mountain region of western Colorado [14], Rocky Mountain National Park, Colorado [15], and the Central Valley of California, USA [16]. Moreover, high mortality of *B. boreas* embryos has been reported in Oregon, USA [17,18]. Western toad populations occur in forest ecosystems [19] and therefore are subject to stressors involved in timber harvest such as habitat loss, habitat fragmentation, road construction, and fertilizer application.

Cascades frogs (*Rana cascadae*) occur throughout the Cascades Mountain Range and breed in temporary ponds or marshes in alpine meadows [12]. Cascades frogs are explosive breeders, typically depositing egg clutches clustered together in shallow areas [12,17,19]. While adults have been observed on land at a distance from water, the overwintering habits of this species are unknown [12]. Some populations of this species appear to be undergoing declines or range reductions [12,17,20].

Long-toed salamanders (*Ambystoma macrodactylum*) occur in a variety of habitats at all elevations in the Pacific Northwest, including desert, forest, woodlands, alpine areas, and agricultural areas [12]. Adults and juveniles are mostly terrestrial, burrow in soil or debris, and likely hunt for prey at night [12]. Migration to ponds for breeding occurs in the autumn at lower elevations and in late winter in the mountains [12]. Long-toed salamanders typically are the first amphibian species to breed at a particular pond and have a brief reproductive period that is spent in the water [12]. Terrestrial-stage long-toed salamanders are associated with older forest ecosystems and may be affected by habitat loss in these habitats [19].

Roughskin newts (*Taricha granulosa*) are also found throughout the Pacific Northwest at most elevations [12]. Roughskin newts are active during the day and occur in a variety of habitats, including valleys, farmland, and conifer forests [12]. They have an extended mating season in which adults remain entirely in the water. The mating season typically begins earlier (e.g., December) and lasts longer at lower elevations than at higher elevations [12]. Roughskin newt eggs are typically attached to vegetation, and larvae usually metamorphose within one season and do not overwinter in water [12]. When out of the water, adult roughskin newts may burrow in soil [21]. Roughskin newts are associated with old-growth forests and are suggested to be at moderate risk of extinction [19,21].

METHODS AND MATERIALS

Animal collection and rearing

Because the four species that we tested breed and metamorphose at different times of the year, our experiments on the four different species were not conduced concurrently. We collected recently metamorphosed (past two weeks) juvenile *B. boreas* at Lost Lake (~74 km east of Sweet Home in Linn County, OR, USA) in October 1999. We collected *R. cascadae* as late-stage tadpoles (Gosner stages 38–43) [22] from Todd Lake (~26 km west of Bend in Deschutes County, OR) and Parish Lake (~62 km east of Lebanon in Linn County, OR) in the Cascades mountain range in August 2000. We reared the Cascades frog larvae in the laboratory on a diet of rabbit chow and TetraMin® (Tetra Sales, Blacksburg, VA, USA) until metamorphosis. We collected *A. macrodactylum* embryos from temporary sites (6 km west and 15 km east of Corvallis in Benton County, OR) in February 2000. The long-toed salamander larvae were reared in the laboratory on a diet of brine shrimp and *Tubifex* worms until metamorphosis. We collected gravid female roughskin newts from the Corvallis watershed (17 km west of Corvallis in Benton County, OR) in April 1999. Eggs were deposited in the laboratory on natural vegetation, and the larvae were reared in the laboratory on a diet of brine shrimp and *Tubifex* worms ad libitum until metamorphosis.

Juvenile (recently metamorphosed) animals were housed in the laboratory in 38-L aquaria until experiments began. Western toads were housed at a density of approximately eight animals per tank; Cascades frogs, long-toed salamanders, and roughskin newts were housed at a density of approximately 15 animals per tank. Western toads were housed at the lower density because previous pilot experiments in our laboratory suggested that they are particularly sensitive to crowding. Approximately 5 L dechlorinated water was available, and aquaria were slanted to provide both a terrestrial and an aquatic surface. The animals were reared under a light:dark regime of 16:8 h using incandescent lighting and a temperature of approximately 20°C. Experiments were conducted during the day under light and temperature conditions similar to those used for rearing. Each experiment for each species was conducted on a separate day

During rearing, juvenile amphibians of all species were fed crickets ad libitum. Each juvenile was used in only one experiment, and animals were assigned to treatments randomly. Mean weights of juveniles prior to the beginning of the experiments for each species were, for Western toads, 0.55 g (standard error [SE] = 0.02 g, $n = 20$); for Cascades frogs, 0.22 g (SE = 0.07, $n = 10$); for long-toed salamanders, 0.42 g (SE = 0.03, $n = 20$); and for roughskin newts, 0.39 g (SE = 0.02, $n = 20$).

Urea fertilizer and dose determination

In all fertilizer treatments, we used granular prilled urea applied by hand (Sim-Plot, Coeur d'Alene, ID, USA). Our treatment levels were equivalent to 50 or 100 kg N/ha. The concentrations of urea were lower than those used in previous

studies in our laboratory [10] and comparable to reported application rates in the field [2,10–11,23]. Oldham et al. [11] report a national average fertilizer application rate of 125 kg/ha in the United Kingdom. Messina [23] reports urea fertilization levels of 230 and 460 kg N/ha in New Zealand. In the Netherlands, mean nitrogen deposition per year is approximately 85 kg N/ha [2]. In the Pacific Northwest, typical forest fertilizer application rates are 150 to 450 kg/ha, and this amount is applied to a plot several times over a 10-year period [10].

We measured nitrate concentration in soil samples that we used after the conclusion of the Western toad experiments. We also collected soil samples from two sites in the Cascades, one where urea fertilizer was applied during the past season (~36 km east of Sweet Home in Linn County, OR; fertilizer applied November 1999; sample collected in March 2000) and one where urea fertilizer was not applied (near Todd Lake, ~26 km west of Bend in Deschutes County, OR). To measure nitrate, we added approximately 40 g soil in 500 ml water, agitated to mix, and then measured the nitrate concentration in the water 48 h later using an Orion pH/ISE nitrate probe (model 290A; Orion Research, Beverly, MA, USA).

Choice test on paper towel: Western toads and Cascades frogs

To test whether juvenile Western toads and Cascades frogs would behaviorally avoid urea, we conducted choice trials using paper towel as substrate. Choice experiments were conducted using rectangular plastic boxes (29 × 16 cm in area, 12 cm deep), divided in half by drawing a line on the outside of the box. New test boxes were used for each experiment. Paper towels were placed in each half of the box, with a 2-cm space between them to minimize diffusion of urea from the treatment side to the control side. For the urea treatments, one paper towel on one half of the box was treated with urea (0.225 g) equivalent to 100 kg/ha. The application of urea on the left or the right side of the box was determined randomly. Control treatments consisted of boxes with plain paper towels on each side. To dissolve the urea and to provide moisture for the Western toads during the experiment, we sprayed water (5 ml) on each half of the box. Spraying both sides ensured that animals would not choose a side based on moisture content. We allowed 3 h for urea to dissolve, then introduced the test animals. We then assigned 15 animals to each treatment by introducing a single animal to the center portion of the box. We allowed animals to acclimate for 10 min and then began observations. We recorded which half of the box animals were located on (whether on the stimulus side or not) once every 10 min for 100 min. Although the control treatment consisted of two water-dosed halves, it was necessary to designate one side as the stimulus side to collect data. We rotated all containers 180° halfway through the experiment to control for any potential geographic bias in animal location.

Choice test on soil: Western toads, Cascades frogs, and long-toed salamanders

To test whether juvenile Western toads, Cascades frogs, and long-toed salamanders would avoid urea on a soil substrate, we conducted choice experiments similar to those described above but using soil rather than paper towel as substrate. We placed approximately 1 L of potting soil (Fred Meyer brand all-purpose potting soil; Fred Meyer, Portland, OR, USA) by volume in a plastic box. The plastic boxes used were of the same dimensions as those used in the paper towel experiment described above. As in the paper towel experiments, new test boxes were used for each experiment. Soil covered the entire bottom surface of the plastic box, so there was no space between the treatment side and the blank side. To separate the treatment side from the control side, we created a neutral strip by placing a 2-cm wide paper towel strip in the middle of the box before urea was added to the treatment side. We removed this strip immediately before animals were added. For the Western toad experiment, treatments included a control and urea at two concentrations, i.e., 0.125 g (equivalent to 50 kg/ha) per one-half box (low) and 0.225 g (equivalent to 100 kg/ha) per one-half box (high). Treatments included a control and high-urea treatment in the Cascades frog and long-toed salamander experiments. We randomly assigned animals (30 in the Western toad experiment, 20 in the Cascades frog and long-toed salamander experiments) to each treatment. We wet the soil by spraying 5 ml water on each side of the box. We allowed six hours for the urea to dissolve completely in the soil (i.e., the urea pellets were no longer visible). The experiment was then started by placing test animals on the neutral strip. We recorded observations as described above for the paper towel choice experiment.

Survival and prey consumption after 5-d exposure: Western toads, Cascades frogs, long-toed salamanders, and roughskin newts

To test the effect of urea on juvenile amphibian survival and feeding rate, we raised juveniles on soil substrates that had either been dosed with urea (urea) or that had been left untreated (control). We added urea to soil in boxes as described above for the choice test on soil except that the boxes were not divided into halves; instead, they contained 1 L of either untreated or urea-dosed potting soil (100 kg/ha). We tested the survival and prey consumption of Western toads, Cascades frogs, long-toed salamanders, and roughskin newts.

The week before the prey consumption experiment, all animals were weighed and then placed in individual plastic boxes that contained damp paper towels as substrate. Juveniles were fed individually to minimize group or competition effects and to ensure that each animal was satiated before testing. After the initial feeding, we randomly assigned animals to treatments (30 per treatment in the Western toad experiment; 20 per treatment for the Cascades frog, long-toed salamander, and roughskin newt experiments). We added urea to the appropriate treatments and sprayed 70 ml of water in each box containing soil the day before adding animals. After adding animals, we assessed juvenile mortality and sprayed 10 ml of water per box to provide moisture each day for 5 d. Animals were not fed during the 5-d exposure.

To assess feeding rate at the end of the 5-d exposure, we placed all juveniles individually in plastic boxes with 15 one-week-old crickets and a damp paper towel substrate (no urea). We counted the number of crickets remaining in each box at the end of a 2-h period and subtracted this number from 15 to determine how many crickets had been eaten.

Data analysis

At the conclusion of the choice experiments, we tallied the number of times each individual was on the stimulus side and compared this value with 50% (five times on stimulus side is expected if choice was random) using the Mann–Whitney rank sum test. We then compared the treatment groups with each

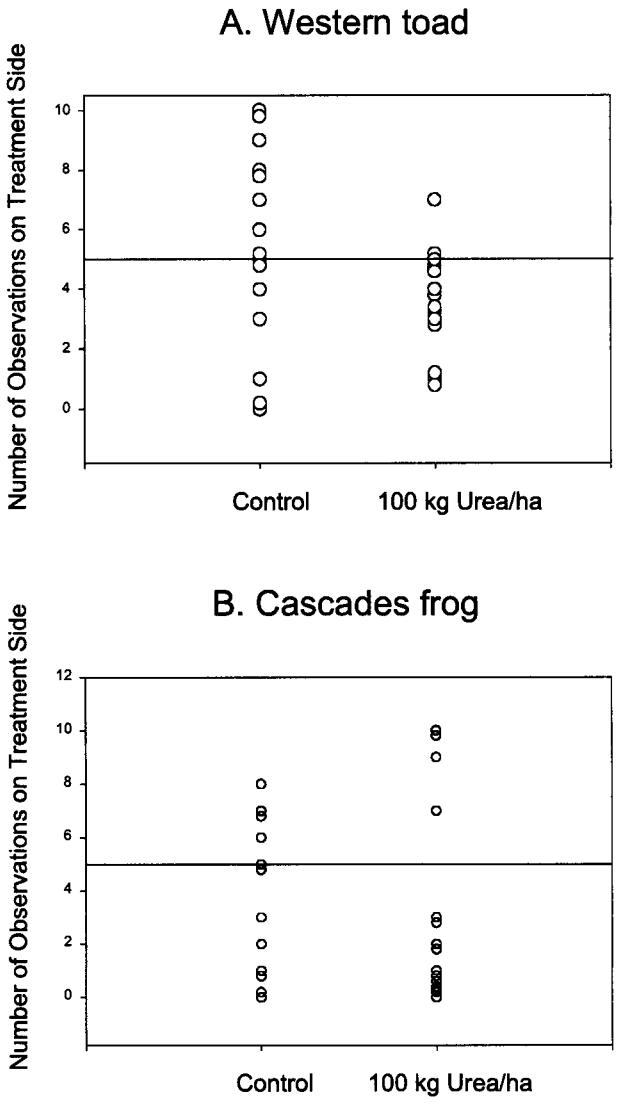

Fig. 1. Choice experiment on paper towel substrate with juvenile Western toads (**A**) and Cascades frogs (**B**). $n = 15$ animals per treatment. Each symbol (dot) represents the response of a single animal. The horizontal line represents the 50% point, expected under random conditions of no choice.

other using the Mann–Whitney rank sum test. To test for differences in survival between the two groups after the 5-d exposure, we used Fisher's exact test [24]. We analyzed for difference in prey consumption between the two treatments using regression analysis so that we could account for weight effects. We set statistical significance at the alpha = 0.05 level.

RESULTS

Choice test on paper towels: Western toads and Cascades frogs

In the Western toad experiment, 7 of 15 animals in the control treatment spent more than half of the observation time on the treatment side, while 1 of 15 Western toads in the urea treatment spent more than half of the observation time on the treatment side (Fig. 1). The control proportion does not differ from the 50% ratio assumed if animals were randomly located (Table 1). In the urea treatment, significantly more Western toads chose the control side over the urea-treated side (Table 1). In the Cascades frog experiment, 6 of 14 animals in the control treatment spent more than half of the observation time on the treatment side, while 4 of 15 Cascades frogs in the urea treatment spent more than half of the observation time on the treatment side (Fig. 1). The control proportion does not differ from the 50% ratio assumed if animals were randomly located (Table 1). In the urea treatment, significantly more Cascades frogs chose the control side over the urea-treated side (Table 1).

Choice test on soil: Western toads, Cascades frogs, and long-toed salamanders

In the Western toad experiment, 11 of 30 Western toads in the high urea treatment spent more than half of the observation time on the treatment side, while 8 of 29 (one escaped) Western toads in the low-urea treatment spent more than half of the observation time on the treatment side. Twelve of 30 control-treatment Western toads spent more than half of the observation time on the treatment side (Fig. 2). The outcome for the control and the high-urea treatments do not differ significantly from what would be expected due to chance alone (Table 1). However, in the low-urea treatment, a significant number of animals avoided the urea side of the exposure container (Table 1). In the Cascades frog experiment, 9 of 20 control animals spent more than half of the observation times on the treatment side, while 7 out of 20 animals in the urea treatment spent more than half of the observation times on the

Table 1. Summary of results of choice tests; Mann–Whitney test compares the treatment to a 50% distribution expected under random (no choice) conditions

	Test		Mann–Whitney rank	
Species	Substrate	Treatment	Sum test statistic	*p*-Value
Western toad	Paper towel	Control	247	0.55
		Urea	172	0.01
Cascades frog	Paper towel	Control	175	0.21
		Urea	180	0.03
Western toad	Soil	Control	870	0.51
		Low urea	725	0.04
		High urea	960	0.51
Cascades frog	Soil	Control	240	0.77
		Urea	263	0.22
Long-toed salamander	Soil	Control	298	0.99
		Urea	272	0.39

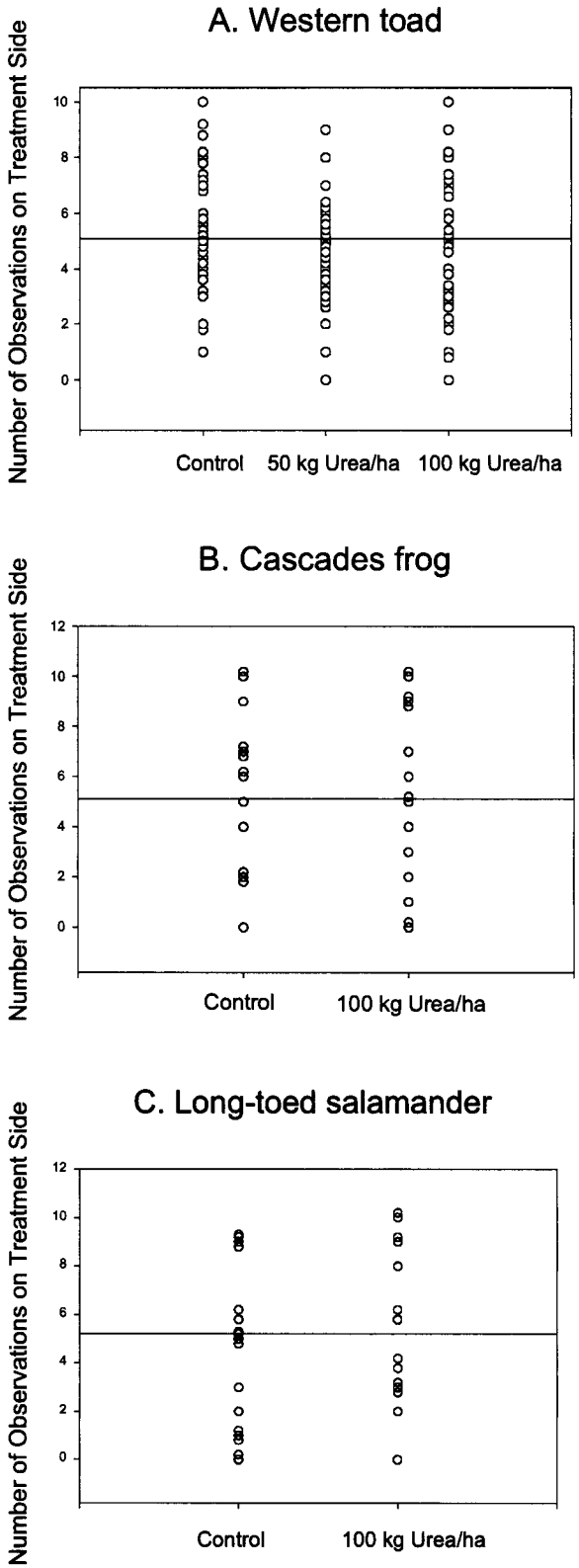

Fig. 2. Choice experiment on soil substrate with juvenile Western toads (n = 30 per treatment) (**A**), Cascades frogs (n = 20 per treatment) (**B**), and long-toed salamanders (**C**) (n = 20 per treatment). Each symbol (dot) represents the response of a single animal. The horizontal line represents the 50% point, expected under random conditions of no choice.

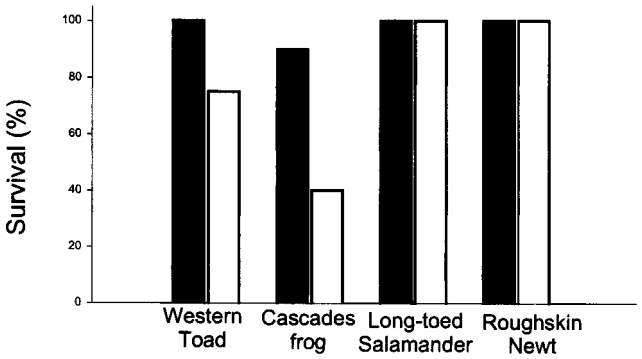

Fig. 3. Survival of juvenile Western toads (n = 30 per treatment), Cascades frogs (n = 20 per treatment), long-toed salamanders (n = 20 per treatment), and roughskin newts (n = 20 per treatment) exposed to urea on soil substrate for 5 d. Control = ■; urea-exposed = □

treatment side (Fig. 2). Neither of these treatments differed from the 50% ratio assumed with random choice (Table 1).

In the experiment with long-toed salamanders, neither of the treatments differed from the 50% ratio expected due to chance alone (Table 1).

Survival of Western toads, Cascades frogs, long-toed salamanders, and roughskin newts on urea-contaminated soil

There was significant mortality of both Western toads and Cascades frogs in the urea treatment when animals were exposed for 5 d on soil (two-tailed Fisher's exact test; $p = 0.041$ for Western toads, $p = 0.002$ for Cascades frogs). In the Western toad experiment, no control animals died, while five urea-exposed Western toads died (Fig. 3). In the Cascades frog experiment, one control animal died and one escaped the experiment; in contrast, 12 urea-exposed frogs died (Fig. 3). No mortality occurred in the long-toed salamander or roughskin newt experiments.

Prey consumption by Western toads, Cascades frogs, long-toed salamanders, and roughskin newts

For both Western toads and Cascades frogs, the proportion of prey consumed increased significantly with weight and decreased with urea exposure (Fig. 4). For Western toads, the effect of urea was marginally significant after accounting for weight ($R^2 = 0.45$, $F_{2,32} = 12.3$, p-value = 0.0001 after accounting for weight; urea T statistic 3.55, p-value = 0.0694; weight T statistic 22.6, p-value = 0.0001). For Cascades frogs, both urea and weight were highly significant in determining prey consumption ($R^2 = 0.39$, $F_{2,23} = 7.2$, p-value = 0.0037 after accounting for weight; urea T statistic 3.16, p-value = 0.0044; weight T statistic 2.75, p-value = 0.0114).

There was no effect of urea on prey consumption in either the long-toed salamander or roughskin newt experiments. However, prey consumption was related to weight in both species; larger animals consumed more crickets (long-toed salamander: $R^2 = 0.17$, $F_{2,38} = 3.7$, p-value = 0.0355 after accounting for weight; urea T statistic 1.24, p-value = 0.2215; weight T statistic 2.42, p-value 0.0207; roughskin newt, $R^2 = 0.18$, $F_{2,28} = 2.91$, p-value = 0.0724; weight T statistic 2.21, p-value 0.0366; urea T statistic 0.93, p-value 0.3604).

Nitrate levels in soil

Nitrate measured from the soil samples was consistent with dose (control, low, high treatments). In the urea-dosed treat-

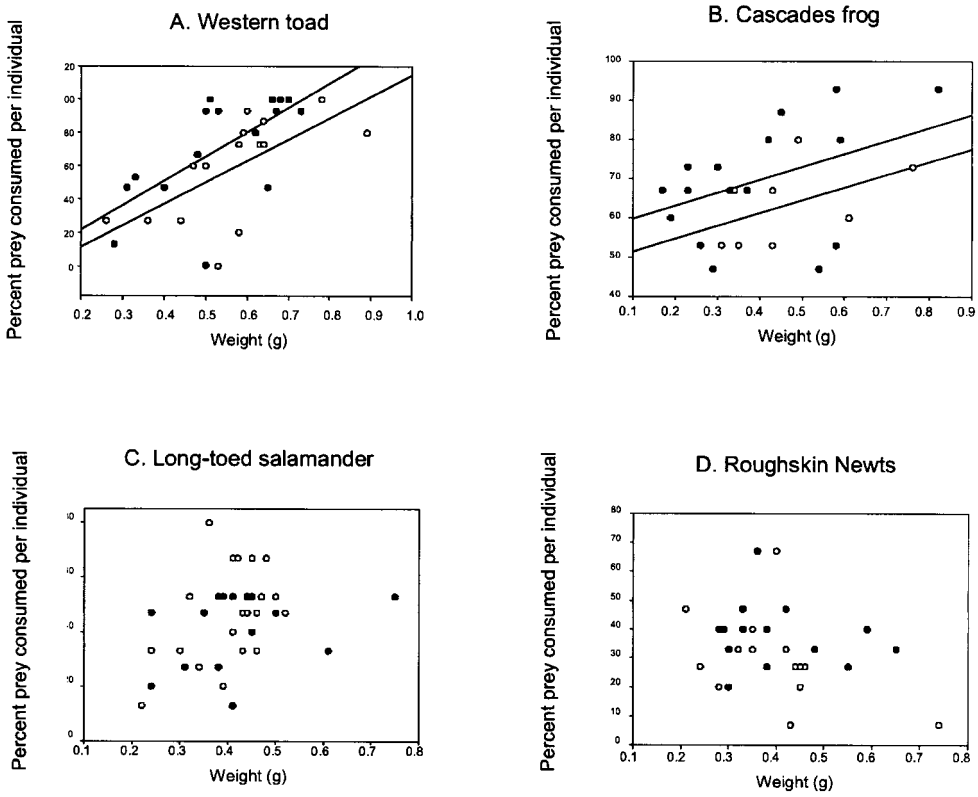

Fig. 4. Prey consumption by juvenile amphibians measured after the animals had been exposed to urea on soil substrate for 5 d. (**A**) Western toads, (**B**) Cascades frogs, (**C**) long-toed salamanders, and (**D**) roughskin newts. For Western toad and Cascades frog, lines represent the regression equation for prey consumption, accounting for weight and urea treatment. Solid symbols (●) represent control animals; open symbols (○) represent urea-exposed animals. Regression lines are presented where urea had a significant effect on prey consumption.

ments from the soil-choice experiment with Western toads, we measured 0.09 mg nitrate/g soil in the low treatment and 0.14 mg nitrate/g soil in the high treatment. In the urea-dosed treatment from the survival and feeding rate exposure with Western toads, we measured 0.11 mg nitrate/g of soil. Control treatments from all experiments yielded nitrate concentrations below the detectable limit (0.01 mg/L). The soil sample from the field site where urea fertilizer had been applied contained 0.001 mg nitrate/g soil, and the soil sample from near Todd Lake (nonimpacted site) yielded a nitrate concentration below the detectable limit.

DISCUSSION

Our study raises several points about the effects of urea on newly metamorphosed amphibians as well as about using terrestrial amphibians in avoidance assays and feeding trials. Understanding the influence that substrate type has on animal behavior is important when considering the effects of contaminants on terrestrial stage-amphibians. Test substrate may influence the ability of juvenile amphibians to detect and respond to stressors such as urea, soil pH, or other variables. In our experiment, the response on a simple substrate (paper towel) was not the same as the response on a more complex substrate (soil), even when exposure to urea via the more complex environment was associated with detrimental effects. Although juvenile Western toads and Cascades frogs had greater mortality and consumed fewer prey when raised on urea-contaminated soil (100 kg/ha) for 5 d, juveniles did not avoid the urea-contaminated soil substrate. In contrast, juvenile Western toads and Cascades frogs did avoid this level of urea on a paper towel substrate. These results suggest that, on natural substrate, juvenile amphibians may not be able to detect and thus be able to behaviorally avoid at least some types of harmful environmental agents. The implication of this result is that, in nature, juvenile terrestrial amphibians may be exposed to environmental contaminants for longer periods of time than they would if they could detect and avoid the contaminant. Steele et al. [25] found that American toad (*Bufo americanus*) tadpoles did not avoid lead-polluted water at levels that had documented adverse effects on larval amphibians (1,000 mg Pb/L), and prior exposure to lead did not affect the discrimination of tadpoles in avoidance trials.

The potential for exposure to urea fertilizer exists for juveniles of all four species tested. All four species inhabit forest ecosystems, where urea is applied to enhance tree growth [10]. In particular, long-toed salamanders burrow in debris or soil, where they might be exposed to urea fertilizers. However, in our experiments with long-toed salamanders and roughskin newts, no negative effects due to urea exposure were evident. In contrast, juveniles of both Western toads and Cascades frogs appeared sensitive to the effects of the urea fertilizer over the 5-d exposure.

In our study, juvenile Western toads were able to avoid contaminated soil at low levels of urea but not at higher levels. There are several possible explanations for this. First, it is possible that, at the higher level of urea, dissolution occurred and the urea filtered into the control side of the experimental container; however, we have no evidence that this occurred. Second, the toxic effects of urea may have altered the activity of the Western toads. Mortality occurred within the 5-d exposure period, so animals could have been stressed by the exposure to urea over the 100-min observation period and were

not able to respond optimally. However, this explanation would not account for why Western toads were not similarly stressed during the choice test on paper towel unless the difference in substrate enabled them to detect the urea more quickly. We are not sure exactly how long it would take for juvenile Western toads to detect and respond to physiological stress from urea in the field.

The mechanism for urea toxicity to amphibians is not entirely known. Osmoregulatory stress could result from urea increasing the salinity of the environment [10] or result in methohemoglutanemia [5]. Urea hydrolysis also may increase soil pH on the forest floor after application, producing toxic ammonia (NH_3) that can easily cross cell membranes and disrupt metabolism in bacteria [26]. Exposure to urea at sublethal levels altered biochemistry and skin composition of the freshwater fish *Oreochromis mossambicus* [27]. Specifically, exposure to 80 ppm urea in the water resulted in significantly increased total free blood sugar levels, increased blood cholesterol level, decreased levels of total sugars in the liver and muscle tissues, decreased protein levels in the liver, and altered histology [27].

Most investigations of contaminant effects on amphibians have focused on the aquatic stages of the amphibian life cycle. Few studies have investigated the effects of environmental contaminants on terrestrial-stage amphibians, perhaps in part because of the difficulty of collecting sufficient sample sizes for experiments [11,28]. However, terrestrial responses and tolerance levels may have a large impact on the distribution and range of amphibians. For example, soil pH affects the distribution of many species of terrestrial salamanders [29].

Feeding rates have been considered a useful toxicological endpoint in several studies [e.g., 7,30], and we suggest that they are particularly useful along with avoidance assays in studies of terrestrial amphibians. Both of these types of assays have the potential to provide information in a relatively short time period so that terrestrial-stage animals do not have to be maintained in the laboratory for long periods of time. Behavioral assays such as these are also ecologically relevant because altered behavior due to stressors may result in an inability of an animal to navigate toward breeding or hydration sites, which could lead to reduced reproductive potential or individual fitness [31]. Exposure to stressors may reduce feeding [e.g., 30], which may ultimately slow growth and affect survival and reproduction.

In our experiments, prey consumption by both Western toads and Cascades frogs was reduced by exposure to urea fertilizer. In all four species tested, we observed a significant trend of increasing weight associated with increased feeding. This trend is similar to that reported in other studies. For example, Newman [32] found that larger toadlets (spadefoot toads, *Scaphiopus couchii*) could more easily capture larger prey. Larger size may be particularly important for foraging success in temporary or crowded environments, where the amount of food per individual is scarce [32].

Avoidance assays are particularly useful in evaluating the effects of environmental stressors on the terrestrial stage of the typical amphibian life cycle because the assay is nondestructive and can reveal significant effects in a relatively short period of time [10]. Choice or avoidance behavior assays are useful indicators of biologically relevant effects in other biological systems [e.g., 33,34]. For example, epibenthic-dwelling amphipods, which are typically sensitive to the effects of environmental contaminants and are not found in heavily contaminated areas, can avoid sediment contaminated with polycyclic aromatic hydrocarbons [33]. Hatch and Burton [35] found that the epibenthic freshwater amphipod *Hyalella azteca* avoided phototoxic effects of the polycyclic aromatic hydrocarbon fluoranthene; they hid in substrate more often in UV-fluoranthene treatments than in treatments with UV alone or fluoranthene alone. In some studies, avoidance behavior is a more sensitive indicator of effects than mortality, revealing significant effects rapidly compared with the length of time needed to examine mortality [33,36]. However, not all types of contaminants can be detected by test organisms, and only those that are detectable by the study organism can be usefully tested in an avoidance assay [36].

Anthropogenic factors undoubtedly are contributing to amphibian population declines at several locations worldwide. Ultraviolet radiation [17,37], global climate change [38], disease [18], and chemical contaminants [39,40] have all been associated with detrimental effects on amphibians that may contribute to population declines. The results of our study and others [3,5,9–11] argue for further study of the impact of nitrogen fertilizers on terrestrial-stage amphibians. The results of our experiments also emphasize the importance of substrate type in avoidance assays for terrestrial amphibians.

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REFERENCES

1. Tilman D. 1999. Global environmental impacts of agricultural expansion: The need for sustainable and efficient practices. *Proc Natl Acad Sci USA* 96:5995–6000.
2. Vitousek PM. 1994. Beyond global warming: Ecology and global change. *Ecology* 75:1861–1876.
3. Marco A, Quilchano C, Blaustein AR. 1999. Sensitivity to nitrate and nitrite in pond-breeding amphibians from the Pacific Northwest, USA. *Environ Toxicol Chem* 18:2836–2839.
4. Marco A, Blaustein AR. 1999. The effects of nitrite on behavior and metamorphosis in Cascades frogs (*Rana cascadae*). *Environ Toxicol Chem* 18:946–949.
5. Rouse JD, Bishop CA, Struger J. 1999. Nitrogen pollution: An assessment of its threat to amphibian survival. *Environ Health Perspect* 107:799–803.
6. Hecnar SJ. 1995. Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from Southern Ontario. *Environ Toxicol Chem* 14:2131–2137.
7. Watt PJ, Oldham RS. 1995. The effect of ammonium nitrate on the feeding and development of larvae of the smooth newt, *Triturus vulgaris* (L.), and on the behavior of its food source, *Daphnia. Freshw Biol* 33:319–324.
8. Blaustein AR. 1994. Chicken little or Nero's fiddle? A perspective on declining amphibian populations. *Herpetologica* 50:85–97.
9. Hatch AC, Blaustein AR. 2000. Combined effects of UV-B, nitrate, and low pH reduce the survival and activity level of larval Cascades frogs (*Rana cascadae*). *Arch Environ Contam Toxicol* 39:494–499.
10. Marco A, Cash D, Belden LK, Blaustein AR. 2001. Sensitivity to forest recommended fertilization doses of urea in three amphibian species from the Pacific Northwest. *Arch Environ Contam Toxicol* 40:406–409.
11. Oldham RS, Latham DM, Hilton-Brown D, Towns M, Cooke AS, Burns A. 1997. The effect of ammonium nitrate fertilizer on frog (*Rana temporaria*) survival. *Agric Ecosyst Environ* 61:69–74.
12. Nussbaum RA, Brodie ED Jr, Storm RM. 1983. *Amphibians and Reptiles of the Pacific Northwest.* University of Idaho Press, Moscow, ID, USA.
13. De Vito J, Chivers DP, Kiesecker JM, Marco A, Wildy EL, Blaustein AR. 1998. The effects of snake predation on metamorphosis of Western toads, *Bufo boreas* (Amphibia, Bufonidae). *Ethology* 104:185–193.

14. Carey C. 1993. Hypothesis concerning the causes of disappearance of boreal toads from the mountains of Colorado. *Conserv Biol* 7:355–362.
15. Corn PS. 1998. Effects of ultraviolet radiation on boreal toads in Colorado. *Ecol Appl* 8:18–26.
16. Fisher RN, Shaffer HB. 1996. The decline of amphibians in California's Great Central Valley. *Conserv Biol* 10:1387–1397.
17. Blaustein AR, Hoffman PD, Hokit DG, Kiesecker JM, Walls SC, Hays JB. 1994. UV repair and resistance to solar UV-B in amphibian eggs: A link to population declines? *Proc Natl Acad Sci USA* 91:1791–1795.
18. Blaustein AR, Hokit DG, O'Hara RK, Holt RA. 1994. Pathogenic fungus contributes to amphibian losses in the Pacific Northwest. *Biol Conserv* 67:251–254.
19. Walls SC, Blaustein AR, Beatty JJ. 1992. Amphibian biodiversity of the Pacific Northwest with special reference to old-growth stands. *Northwest Environ J* 8:53–69.
20. Fellers GM, Drost CA. 1993. Disappearance of the Cascades frog *Rana cascadae* at the southern end of its range, California, USA. *Biol Conserv* 65:177–181.
21. Blaustein AR, Beatty JJ, Olson DH, Storm RM. 1995. The biology of amphibians and reptiles in old-growth forests in the Pacific Northwest. PNW-GTR-337. General Technical Report. U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Portland, OR.
22. Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183–190.
23. Messina MG. 1992. Response of *Eucalyptus regnans* F. Muell. to thinning and urea fertilization in New Zealand. *For Ecol Manag* 51:269–283.
24. Zar JH. 1999. *Biostatistical Analysis.* Prentice Hall, Princeton, NJ, USA.
25. Steele CW, Strickler-Shaw S, Taylor DH. 1991. Failure of *Bufo americanus* tadpoles to avoid lead-enriched water. *J Herpatol* 25: 241–243.
26. Kernaghan G, Berch S, Carter R. 1995. Effect of urea fertilization on ectomycorrhizae of 20-year-old *Tsuga heterophylla*. *Can J For Res* 25:891–901.
27. Balasubramanian P, Saravanan TS, Palaniappan MK. 1999. Biochemical and histopathological changes in certain tissues of *Oreochromis mossambicus* (Trewaves) under ambient urea stress. *Bull Environ Contamin Toxicol* 63:117–124.
28. Hopkins WA, Mendoca MT, Rowe CL, Congdon JD. 1998. Elevated trace element concentrations in Southern toads, *Bufo terrestris*, exposed to coal combustion waste. *Arch Environ Contamin Toxicol* 35:325–329.
29. Horne MT, Dunson WA. 1994. Behavioral and physiological responses of the terrestrial life stages of the Jefferson salamander, *Ambystoma jeffersonianum*, to low soil pH. *Arch Environ Contamin Toxicol* 27:232–238.
30. Hatch AC, Burton GA Jr. 1999. Phototoxicity of fluoranthene to two freshwater crustaceans, *Hyalella azteca* and *Daphnia magna*: Measures of feeding inhibition as a toxicological endpoint. *Hydrobiologia* 400:243–248.
31. Blaustein AR, Chivers DP, Kats LB, Kiesecker JM. 2000. Effects of ultraviolet radiation on locomotion and orientation in roughskin newts (*Taricha granulosa*). *Ethology* 106:227–234.
32. Newman RA. 1999. Body size and diet of recently metamorphosed spadefoot toads (*Scaphiopus couchii*). *Herpetologica* 55: 507–515.
33. Kravitz MJ, Lamberson JO, Ferraro SP, Swartz R. 1999. Avoidance response of the estuarine amphipod *Eohaustorius estuarius* to polycyclic aromatic hydrocarbon-contaminated, field-collected sediments. *Environ Toxicol Chem* 18:1232–1235.
34. Lotufo GR. 1997. Toxicity of sediment-associated PAHs to an estuarine copepod: Effects on survival, feeding, reproduction and behavior. *Mar Environ Res* 44:149–166.
35. Hatch AC, Burton GA Jr. 1999b. Photo-induced toxicity of PAHs to *Hyalella azteca* and *Chironomus tentans*: Effects of mixtures and behavior. *Environ Pollut* 106:157–167.
36. Yeardley RB Jr. 1996. The potential of an earthworm avoidance test for evaluation of hazardous waste sites. *Environ Toxicol Chem* 15:1532–1537.
37. Blaustein AR, Kiesecker JM, Chivers DP, Hokit DG, Marco A, Belden LK, Hatch AC. 1998. Effects of ultraviolet radiation on amphibians: Field experiments. *Am Zool* 38:799–812.
38. Pounds JA, Fogden MPL, Campbell JH. 1999. Biological response to climate change on a tropical mountain. *Nature* 398: 611–615.
39. Hatch AC, Burton GA Jr. 1998. Effects of photoinduced toxicity of fluoranthene on amphibian embryos and larvae. *Environ Toxicol Chem* 17:1777–1785.
40. Howe GE, Gillis R, Mowbray RC. 1998. Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae. *Environ Toxicol Chem* 17:519–525.