ARE INDUCED DEFENSES COSTLY? CONSEQUENCES OF PREDATOR-INDUCED DEFENSES IN WESTERN TOADS, BUFO BOREAS

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Abstract. Induced defenses are widespread in nature, and in amphibian larvae they are often expressed as altered behavior and changes in tail shape, color, and size. Theory predicts that induced defenses should be costly in the absence of a predator threat. No costs have been found for these defenses after metamorphosis. In this study, we tested for induced defenses in western toads, Bufo boreas, and measured larval and postmetamorphic consequences of these responses. Larvae were raised in either the presence or absence of nonlethal predator cues. Defense responses to these larval treatments were measured during the larval stage and shortly after metamorphosis using both predator bioassays and quantification of the putative chemical defense common in toads, bufadienolides. We found no differences in larval morphology, growth rate, or development rate between the predator and control treatments. In the larval bioassays, some types of invertebrate predators consumed significantly fewer of the B. boreas larvae that were reared with predator cues compared to the control treatments. Bufadienolides were not present in B. boreas larvae. In the postmetamorphic bioassays, tiger salamanders (Ambystoma tigrinum) had longer handling times when consuming B. boreas that had developed in larval environments without predator cues compared to predator-treatment B. boreas. However, postmetamorphic B. boreas from predator cue larval environments had significantly higher concentrations of bufadienolides than did those from larval environments without predators, suggesting that these defenses are ineffective against tiger salamanders. Our results demonstrate that there is plasticity in the chemical defenses of toads and suggest that induced larval defenses may incur costs that are only apparent after metamorphosis.

Key words: Ambystoma tigrinum; amphibian larvae; bufadienolides; Bufo boreas; chemical defense; cost; inducible defense; phenotypic plasticity; predator; tiger salamander; western toad.

INTRODUCTION

Ecologists are becoming increasingly aware of the important role that induced defenses play in ecological interactions. For example, inducible defenses have become a paradigm in the field of plant–herbivore interactions (Karban and Baldwin 1997, Agrawal 1999). Theory predicts that for an inducible defense to be an evolutionary stable strategy, the induced phenotype must have lower fitness than the alternate or constitutive phenotype in the absence of predation risk (Lively 1986, 1999, Clark and Harvell 1992). For example, changes in body depth in Crucian carp (Carassius carassius) in response to predator cues result in increased energetic swimming costs due to increased drag (Brönmark and Miner 1992, Pettersson and Bronmark 1997). Similarly, the predator-induced spines of many cladocera are effective at reducing predator threat, but can increase metabolic costs of swimming or result in smaller offspring that are more susceptible to starvation (Tollrian and Dodson 1999). Thus, predator-induced plasticity can benefit individuals in the presence of legitimate risk, but in the absence of such risk, the induced defense (in these examples, morphology) may result in a fitness cost.

Many amphibian larvae exhibit plasticity in behavior, morphology, developmental rate, and growth rate in response to environmental conditions (Wilbur and Collins 1973, Skelly and Werner 1990, Skelly 1992, Newman 1994, Chivers et al. 1999, Relyea and Werner 1999, Lardner 2000). For example, cues indicating conspecific density or larval habitat permanency can induce plastic responses. These can include facultative cannibalism, which results in changes in head and digestive tract morphology (Pfennig 1992, Walls et al. 1993), or facultative paedomorphosis, in which individuals become sexually mature while retaining their aquatic larval phenotype (Semlitsch et al. 1990). Similarly, natural enemy cues can induce changes in tail shape in a way that promotes escape from predators (McCollum and Van Buskirk 1996, Lardner 2000, Relyea 2001a, b). Plasticity in morphology, growth, and development are often associated with changes in foraging behavior or microhabitat use (Skelly and Werner 1990, Relyea and Werner 1999). These different strategies for coping with different environmental conditions during the larval period have been shown to be adaptive in each case where they have been examined (Pfennig 1992, Newman 1994, McCollum and Van Buskirk 1996, Van Buskirk et al. 1997). In contrast to...
the many studies investigating plasticity in morphology, behavior, and developmental growth rate in larval amphibians, no studies have examined plasticity in chemical defenses. Chemical defenses are common and diverse in amphibians, and have played an important role in their evolutionary history (Flier et al. 1980, Daly 1995, Toledo and Jared 1995). It has yet to be established if amphibians modify their investment in chemical defenses in response to environmental cues, including cues indicating predation risk (i.e., inducible chemical defenses).

Most investigations of the fitness cost of predator-induced responses have emphasized their effect on larval survival and growth. For instance, McCollum and Van Buskirk (1996) found that the predator-induced change in tail morphology, which improved the ability of gray treefrog (Hyla chrysoscelis) larvae to escape insect predators, reduced H. chrysoscelis survival and size at metamorphosis in the absence of predators. Although such studies demonstrate definite costs incurred during the larval period, they do not specifically investigate postmetamorphic costs. However, larval environment (food, temperature, presence of predator cues) has been shown to influence size and relative limb lengths in several anurans at metamorphosis, indicating that the consequences of induced defenses may also be carried past metamorphosis (Goater et al. 1993, Beck and Congdon 2000, Blouin and Brown 2000, Reylea 2001c). Van Buskirk and Saxer (2001) tested for, but found no immediate postmetamorphic costs for the predator-induced defenses of European water frogs, Rana ridibunda. Significant cost of induced larval defense could be present if the allocation of resources for larval defense traits results in reduced adult growth or defense. Alternatively, if larvae and adults use the same defense mechanism, such as toxic chemical defense, newly metamorphosed individuals may actually benefit from an increased larval investment in these chemical defenses.

In this study, we investigated the larval and postmetamorphic consequences of cues indicating the presence of predators in the larval period of western toads, Bufo boreas (Bufoidae). Toads are well known to be chemically defended. One important group of toxins which are toxic to many predators (Whittaker and Feeny 1971, Flier et al. 1980) are bufadienolides, which are toxic to many predators (Whittaker and Feeny 1971, Flier et al. 1980, Toledo and Jared 1995). It is not known, however, if these compounds function as larval defenses or if larval conditions influence postmetamorphic investment in these compounds. Bioassays have indicated that there is an ontogenetic change from nontoxic to toxic, or palatable to unpalatable, in many Bufo larva (Brodie et al. 1978, Kruse and Stone 1984, Brodie and Formanowicz 1987, Crossland 1998). Using both bioassays and analytical measurements of bufadienolide content, we tested (1) if predator cues cause Bufo boreas larvae to induce a defense, (2) if there is a cost during the larval period to inducing or maintaining a defense, and (3) if there is a cost after the larval period to inducing or maintaining the defense.

**METHODS**

**Natural history of Bufo boreas**

*Bufo boreas* ranges from northern Baja California to southern Alaska, and from the Pacific coast as far east as Colorado, United States (Stebbins 1985). Adults are terrestrial except during the breeding season. Females lay eggs in ponds and other bodies of water during winter or spring rains. *Bufo boreas* larvae are preyed upon by a variety of predators, including salamanders, frogs, birds, garter snakes, and invertebrates (Arnold and Wassersug 1978, Beiswenger 1981, Hews 1988, Blaustein et al. 1990, Peterson and Blaustein 1992, Kiesecker et al. 1996). Larval *B. boreas* have been shown to be unpalatable to roughskin newts (*Taricha torosa*) and northwestern salamanders (*Ambystoma gracile*), although *B. boreas* larvae at all developmental stages were palatable to two invertebrate predators (Peterson and Blaustein 1991, 1992). Two studies have demonstrated that *B. americanus* and *B. marinus* larvae increase in unpalatability or toxicity to some predators as they approach metamorphosis (Brodie et al. 1978, Crossland 1998). Larval *B. boreas* exhibit altered behavior and reduced duration of larval period, but not size at metamorphosis, when raised in the nonlethal presence of predator cues (Hews 1988, Kiesecker et al. 1996, Chivers et al. 1999). *Bufo americanus* reduce their movement and metamorphose at a smaller size in the presence of predator cues (Skelly and Werner 1990). Only one study has tested for morphological plasticity in response to predator cues in a *Bufo* species, but they did not detect a change in tadpole shape (Lardner 2000). *Bufo boreas* may face heavy mortality immediately after metamorphosis from frog and garter snake predators (Arnold and Wassersug 1978, Pearl and Hayes 2002).

**Experimental design**

We collected *B. boreas* larvae from across a large subdivided pond in Elverta, California (Sacramento County) on 14 April 2000. Based on the size of the pond and the number of tadpoles, we estimate that more than five families were collected. Each experimental cohort consisted of 10 individuals of the same approximate size and developmental stage (Gosner 1960: Gosner Stage 29). Larvae were maintained on a diet of ground Kaytee Supreme Daily Blend Rabbit Pellets (main ingredients: alfalfa meal, wheat middling, ground grain products; Kaytee, Chilton, Wisconsin, USA) using the formula of Alford and Harris (1988) to assign an intermediate amount of food. Our experimental procedures are illustrated as a flowchart in Fig. 1. In total, we established 150 experimental cohorts in separate rectangular containers in an animal care fa-
Fig. 1. Flow chart illustrating the experimental procedure of predator and control treatments for western toad (*Bufo boreas*) larvae.

- **Mid-larval bioassay**
  1. Remove all larvae from 25 predator cohorts and 21 control cohorts.
  2. Measure all larvae from those cohorts.
  3. Put four randomly chosen larvae from each cohort in a cup with predators: 12 predator and 10 control cohorts go to *Dytiscus*, and 13 predator and 11 control cohorts go to *Aeshna*.
  4. Freeze remaining larvae from each cohort for HPLC analysis.

- **Late-larval bioassay**
  1. Remove all larvae from 20 predator cohorts and 19 control cohorts.
  2. Measure all larvae from those cohorts.
  3. Put three randomly chosen larvae from each cohort in a cup with predators: 9 predator and 12 control cohorts go to *Abedius*, and 11 predator and 8 control cohorts go to *Aeshna*.
  4. Freeze remaining larvae from each cohort for HPLC analysis.

- **Metamorphosis**
  1. Weigh all metamorphs from the remaining cohorts and record date.
  2. Freeze second metamorph from each cohort for HPLC analysis.

- **Postmetamorphic bioassay** (20 trials)
  1. Put one metamorph in with a tiger salamander.
  2. Record time until metamorph is eaten/or 24 hours.
  3. Record handling time when metamorph is eaten.
  4. Feed cricket to tiger salamander.
  5. Freeze 12 separate *B. boreas* metamorphs on the day of the terrestrial bioassay for HPLC analysis.

Cohorts were raised in a facility at University of California, Davis (12:12 L:D, temperature 19–24°C). Each container was 30 cm long and 15.5 cm wide, filled with artificial pond water (25% Hoftreter’s solution) to a depth of 4.5 cm. We maintained each cohort at a constant water level and changed the water every 10 to 12 d. Within 48 h after collecting the *B. boreas*, each replicate was assigned a priori as a control or to receive predator cues and each was assigned to one of three assay dates (mid-larval, late-larval, postmetamorphic). Predator cues were introduced every other day by the addition of 0.2 g of homogenized *B. boreas* larvae suspended in 25 mL of artificial pond water. This method of providing predator cues has been shown to have effects on development equivalent to providing chemical cues of an invertebrate predator feeding on *B. boreas* larvae (Chivers et al. 1999). Control replicates were given a sham control of the same volume of artificial pond water at the same time that the predator cues were applied. To determine if larvae raised in the presence of predator cues were more likely to survive in the presence of predators, we performed biological assays, which we will describe in detail, on the larvae at three points in development: mid-larval, late-larval, and postmetamorphosis. To test
for plasticity in the putative anti-predator toxin, bufadienolides, we sampled individuals from each replicate during these three developmental points for HPLC (high-performance liquid chromatography) analysis. We also tested for predator-induced plasticity in cohort mass, individual body length, tail length, tail height, and tail muscle height. Statistical analyses were performed with the JMP IN 4.0.1 statistical package (Sall et al. 2001).

**Growth and metamorphosis**

All cohorts not used in the two bioassays were raised through metamorphosis. We measured the mass of each *Bufo boreas* once it reached Gosner stage 42 (emergence of first forelimb from operculum) and then allowed it to complete metamorphosis in a container with a base that sloped out of the water. Once most larvae reached terrestrial form, they were placed in a cup with wet paper towels and were fed fruit flies ad libitum. Once the second metamorph from each replicate reached the terrestrial stage (with the tail completely absorbed), we froze it at −80°C for later bufadienolide analysis.

**Bioassays**

Our motivation for conducting the bioassays was to determine if the larval environment would affect *B. boreas* defense against predators, measured either as predator rejection or increased handling times. Each cohort used in a bioassay was weighed as a group and the number of surviving larvae was counted. We used digital calipers to measure the body length, tail length, tail height, and tail muscle height of each individual larva. We tested for differences in morphology between the predator and control treatments with a MANOVA on all measured morphological traits and mass. Four larvae from each experimental cohort were haphazardly assigned to the bioassays and the remaining were frozen at −80°C until bufadienolide analysis.

Bioassays on the tadpoles were performed using invertebrate predators. The mid-larval bioassays were performed with predaceous diving beetle larvae (*Dytiscidae: Dytiscus* sp.) and dragonfly nymphs (*Aeshnidae: Aeshna* sp.) on day 21 of the experiment (16 May). The late-larval bioassays were performed with giant water bugs (*Bellostomatidae: Abedus indentatus*) and *Aeshna* sp. on day 48 of the experiment (13 June). We used the *A. indentatus* as an alternative to *Dytiscus* because the *Dytiscus* larvae were not available at that time of year. These are all natural predators of *B. boreas*. Each predator was used only once. The *Dytiscus* larvae were obtained from the Quail Ridge Reserve (Napa County, California, USA), part of the University of California Natural Reserve System. The *Aeshna* and *A. indentatus* were obtained from Stebbins Cold Canyon Reserve (Solano County, California) also part of the University of California Natural Reserve System in Solano County, California. *Bufo boreas* are found at both localities. Bioassays were conducted in arenas consisting of a clear plastic cup (5 cm diameter) filled with 5 cm of artificial pond water. Each of the predators was acclimated to an arena for a day preceding the bioassay. *B. boreas* larvae from an experimental cohort were placed in a predetermined arena with a predator. In the mid-larval bioassay, we used four *B. boreas* larvae per arena, and in the late-larval bioassay, we used three *B. boreas* larvae per arena. In the mid-larval assay, each arena was observed every 7 min for 400 consecutive minutes to record the number of larvae surviving in each arena as a measure of *B. boreas* putative defense. In the late-larval assay, each arena was observed every 7 min for 350 min. Comparisons of predator- and control-treatment cohort survivorship of larvae were carried out using Kaplan-Meier survival analysis. Survival data from each of the predator experiments were analyzed separately. To further test for evidence of an induced chemical defense, we examined the duration of time that each invertebrate predator took to consume the third tadpole eaten (in the first larval bioassay) or the second tadpole eaten (in the second larval bioassay). If there is an induced chemical defense in the tadpoles reared with nonlethal predator cues, it may have an observable effect on the predator after the predator has eaten one tadpole.

The postmetamorphic bioassay was performed on day 71 (6 August). The predators that we used were recently metamorphosed tiger salamanders, *Ambystoma tigrinum*, collected as larvae from a population in Lake County, California, well within the natural range of *B. boreas*. Prior to the bioassay, these salamanders were maintained on a diet of crickets. Although collected from a reproducing population in Lake County, California, these animals are derived from an introduced set of populations from the Great Plains that was established in the 1950s (H. B. Shaffer, personal communication). The *A. tigrinum* were not fed for one week prior to the bioassay.

In the bioassays, each *A. tigrinum* was presented with one *B. boreas* that had been the first to metamorphose from its experimental cohort. We performed 10 standardized trials for each treatment. The *B. boreas* was placed 3–5 cm in front of the *A. tigrinum*. After each *A. tigrinum* struck, we recorded whether it rejected or swallowed the *B. boreas*. We considered it a rejection if the *A. tigrinum* bit the toad, held it, released it, and then showed no interest in it for 2 min after the toad began moving again. The choice of 2 min was based on observations of the behavior of captive *A. tigrinum* when feeding on crickets. If *A. tigrinum* did not show interest in crickets within two min, they usually would not eat the crickets until at least the following day. In the cases in which *A. tigrinum* swallowed the *B. boreas*, we measured how long it took them to swallow the toad. To determine if eating *B. boreas* from either the control or predator cue treatment affected the subsequent foraging efficiency of *A. tigrinum*, possibly through a toxic effect of toad chemical defense, we fed...
under laboratory conditions, we collected 15 test for possible confounding effects of development each bioassay and on individuals at metamorphosis. To HPLC analysis on individuals sampled at the time of summed a B. boreas Massachusetts, USA). Each injection was 10 \( \mu \)L, eluted (60:40) at 1.25 mL/min on a 250-4 LiChroCART RP-18 column packed with 5 \( \mu \)m LiChrospher 100 (E. Merck, Darmstadt, Germany), with a 10-mm guard column packed with the same material. This methodology is similar to that described by Gella et al. (1995). Identification of bufadienolides was based upon the absorption spectra compared to known standards obtained from Sigma Scientific (Sigma, Houston, Texas, USA). A dilution series of bufalin and cinobufagin was used to establish a calibration curve to quantify the bufadienolide content of each sample. This dilution series indicated that our method was able to reliably quantify concentrations of bufadienolide as low as 0.00025 mg/1 mL of solution. We compared the treatments using an ANCOVA comparing the total amount (in micrograms) of bufadienolides in each sample with dry mass as the covariate.

**HPLC assays**

To determine the possible role of bufadienolides in B. boreas encounters with predators, we performed HPLC analysis on individuals sampled at the time of each bioassay and on individuals at metamorphosis. To test for possible confounding effects of development under laboratory conditions, we collected 15 B. boreas larvae from the original source pond at approximately Gosner stage 30 and 35. These B. boreas were frozen at the time of their capture and stored at \(-80^\circ\)C until HPLC analysis of bufadienolides.

**Bufo boreas** samples were dried under reduced pressure. Each dried sample was weighed and crushed with a glass rod. Each sample was defatted in 10 mL of hexane. Analysis of hexane extract revealed that no bufadienolides were present in the hexane extract. Each defatted sample received 7 mL of methanol and was sonicated for 20 min. After 5 min of high-speed centrifuging, the supernatant was placed in a new tube and the procedure was repeated on the tissue sample. The methane was evaporated by placing the tubes in a warm-water bath under nitrogen. The residue of each extract was resuspended in 1 mL of methanol and passed through a 0.45-\( \mu \)m filter into a 1-mL autosampler vial for HPLC analysis. HPLC analyses were performed with a Waters HPLC system with WISP autosampler, 600E pump, 996 photodiode array detector (at 300 nm) using Millennium 2010 chromatography manager software (all equipment from Waters, Milford, Massachusetts, USA). Each injection was 10 \( \mu \)L, eluted isocratically with a mixture of water and acetonitrile (60:40) at 1.25 mL/min on a 250-4 LiChroCART RP-18 column packed with 5 \( \mu \)m LiChrospher 100 (E. Merck, Darmstadt, Germany), with a 10-mm guard column packed with the same material. This methodology is similar to that described by Gella et al. (1995). Identification of bufadienolides was based upon the absorption spectra compared to known standards obtained from Sigma Scientific (Sigma, Houston, Texas, USA). A dilution series of bufalin and cinobufagin was used to establish a calibration curve to quantify the bufadienolide content of each sample. This dilution series indicated that our method was able to reliably quantify concentrations of bufadienolide as low as 0.00025 mg/1 mL of solution. We compared the treatments using an ANCOVA comparing the total amount (in micrograms) of bufadienolides in each sample with dry mass as the covariate.

**RESULTS**

**Growth and metamorphosis**

All values are expressed as mean ± 1 SE. There was no significant effect of larval treatment on mass at metamorphosis (control treatment, \( n = 18 \), 1.50 ± 0.45 g; predator treatment, \( n = 17 \), 1.12 ± 0.46 g) or on time to metamorphosis (control, \( n = 18 \), 65.91 ± 0.61 d; predator, \( n = 17 \), 64.75 ± 0.63 d; MANOVA, \( F_{1,32} = 0.915, P = 0.410 \)). The number of larvae surviving until the day that the first individual metamorphosed did not differ between predator treatments (\( n = 18 \), 4.33 ± 0.49 larvae) and control treatments (\( n = 19 \), 4.21 ± 0.49 larvae; unpaired \( t \) test, \( df = 35 \), \( t = 0.211 \), \( P = 0.833 \)).

**Mid-larval assay**

There was no detectable difference in morphology between the control and the predator treatments (Table 1). None of the **Bufo boreas** larvae had detectable bufadienolides. Larvae in this assay were, on average, Gosner stage 31.

There was no difference between the treatments in the rate at which **Bufo boreas** larvae were consumed by the *Aeshna* (Fig. 2A; \( n = 24 \), \( df = 1 \), \( \chi^2 = 0.434, P \)
FIG. 2. Survival plot for *Bufo boreas* larvae in each arena in the mid-larval bioassay. (A) *Aeshna* (dragonfly nymphs) consumed *B. boreas* at the same rate irrespective of treatment (Mantel-Cox, $\chi^2 = 0.434$, df = 1, $P = 0.51$). (B) *Dytiscus* (predaceous diving beetle larvae) consumed *B. boreas* from predator treatments significantly faster than they consumed *B. boreas* from control treatments (Mantel-Cox, $\chi^2 = 4.524$, df = 1, $P = 0.033$).

TABLE 2. MANOVA for the effects of treatment (predator vs. control) on tail height, tail length, tail muscle height, body length, and mass of western toads at the time of the late-larval assay (Gosner Stage 35).

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</table>

FIG. 3. Survival plot for *B. boreas* larvae in each arena in the late-larval bioassay. (A) *Aeshna* (dragonfly nymphs) consumed *B. boreas* from predator treatments significantly faster than they consumed *B. boreas* from control treatments (Mantel-Cox, $\chi^2 = 3.928$, df = 1, $P = 0.048$). (B) *Abedus indentatus* (giant water bugs) consumed *B. boreas* at the same rate, irrespective of treatment (Mantel-Cox, $\chi^2 = 0.800$, df = 1, $P = 0.371$).

Late-larval assay
As in the mid-larval assay, we found no difference in mass or tail shape of *B. boreas* between the predator treatment and the control treatment (Table 2), nor did we detect bufadienolides in the *B. boreas* larvae. Larvae in this assay were, on average, Gosner stage 35.

Unlike the mid-larval assay, the *Aeshna* response differed between the two treatments. In the late-larval assay, *Aeshna* consumed all of the control-treatment *B. boreas* in only 110 min, whereas at the end of the 350 min, 35% of the predator-treatment *B. boreas* cohorts had surviving larvae (Fig. 3A; $n = 16$, df = 1, $\chi^2 = 3.928$, $P = 0.048$). In the *Abedus* bioassay, however, there were no differences in *B. boreas* survivorship between the predator treatment and the control treatment (Fig. 3B; $n = 20$, df = 1, $\chi^2 = 0.800$, $P = 0.371$). There was no difference in the time that it took *Aeshna* or *Abedus* to consume the second *B. boreas* from either treatment (ANOVA for *Aeshna*, $F_{1.10} = 0.31$, $P = 0.59$; ANOVA for *Abedus*, $F_{1.16} = 0.2.98$, $P = 0.10$).

Postmetamorphic assay
There was no difference in mass of the *B. boreas* between the two treatments (control treatment, 0.38 ± 0.03 g; all data are presented as mean ± 1 se, predator treatment, 0.37 ± 0.03 g; ANOVA, $F_{1.18} = 1.056$, $P = 0.815$). In each treatment, six *A. tigrinum* swallowed
the *B. boreas* and four *A. tigrinum* rejected the *B. boreas*. All *A. tigrinum* survived after swallowing the *B. boreas*. The *A. tigrinum* took significantly longer to swallow toads that had been raised in control treatments than those raised with predator cues (Fig. 4A, Table 3).

There was no effect of larval *B. boreas* treatments on the amount of time that it took each *A. tigrinum* to subsequently strike at the cricket (Fig. 4B, Table 4). There was also no effect of toad mass on the time that it took each *A. tigrinum* to first strike at a cricket, but larger *A. tigrinum* had earlier first strikes than smaller *A. tigrinum* (Table 4). However, *A. tigrinum* took three times as long to catch a cricket if they had eaten *B. boreas* from control treatments rather than predator treatments (Fig. 4C, Table 5).

The *B. boreas* that were frozen immediately after metamorphosis contained either no bufadienolides (15 toads in the predator treatment, 14 in the control treatment), or only trace amounts of bufadienolides that were not quantifiable (two toads in the predator treatment, four in the control treatment). However, we found quantifiable amounts of bufadienolides in the *B. boreas* that we raised until the day of the postmetamorphic bioassay. At least 20 d had elapsed from the time that these individuals had metamorphosed until they were frozen. *Bufo boreas* in the predation treatment had a threefold higher total amount of bufadienolides than those in the control treatment (Fig. 5; ANCOVA, $F_{2,9} = 8.720, P = 0.0161$).

**Buofadienolide analysis of wild-caught *B. boreas***

The *B. boreas* larvae collected after several weeks of development in their natural pond were approximately Gosner stage 30 in the first collection and Gosner stage 35 in the second collection. These match the Gosner stages of our laboratory-raised animals. We did not detect bufadienolides in individuals from either of these samples.

**DISCUSSION**

Our results indicate that *Bufo boreas* larvae did induce a defense in response to predator cues and that this induced defense was effective against some of the predators. We did not detect morphological plasticity in response to predator cues, or the development of bufadienolide toxin in larvae of either treatment. However, because multiple bioassays demonstrated that *B. boreas* larvae raised in the presence of predator cues had a higher probability of survival than did larvae in the control treatments, we infer that there was some induced defense. We are unable to fully explain the

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**Table 3. ANCOVA for the effects of larval treatment on the time that it took for each adult salamander to consume a western toad, once captured.**

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</table>

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**Table 4. ANCOVA of the effects of larval treatment on the time that it took each adult salamander to first strike at a cricket placed into its cage, one hour after it swallowed a toad.**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>ss</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>41.37</td>
<td>3.67</td>
<td>0.0711</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>26.30</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>5.41</td>
<td>1.44</td>
<td>0.2690</td>
</tr>
<tr>
<td>Predator mass</td>
<td>1</td>
<td>20.76</td>
<td>5.56</td>
<td>0.0510</td>
</tr>
<tr>
<td>Toad mass</td>
<td>1</td>
<td>0.07</td>
<td>0.018</td>
<td>0.8983</td>
</tr>
</tbody>
</table>
mechanism that caused the *Dytiscus* in the mid-larval assay and the *Aeshna* in the late-larval assay to take longer to consume all of the predator-treatment larvae compared to the control-treatment larvae. All other studies of induced defense in amphibians have found a correlation between induced morphology and survival (McCollum and Van Buskirk 1996, Van Buskirk et al. 1997, Van Buskirk and Schmidt 2000, Relyea 2001a, b). However, in our study there was no difference in overall morphology or mass between treatments. Therefore, we can rule out these traits as an explanation of treatment differences in survival. Additionally, a difference in activity level between predator-treatment and control *B. boreas* larvae is an unlikely explanation for the observed difference in the responses of the predators, because nearly all of the *B. boreas* larvae stopped moving once the predator had eaten the first *B. boreas* larvae.

One potential explanation is that the predator-cue treatment induced an increased production of a non-bufadienolide toxin that caused the predators to consume the predator-treatment *B. boreas* more slowly than the control-treatment *B. boreas*. Supporting this hypothesis, *Dytiscus* larvae that were fed the predator-treatment *B. boreas* took significantly longer to consume a second tadpole after having eaten one tadpole than did *Dytiscus* fed control-treatment larvae. This is consistent with the *Dytiscus* being slowed by a toxin. Besides bufadienolides, other toxins have been found in different *Bufo* species, including biogenic amines and morphine (Toledo and Jared 1995). The implication of toxins other than bufadienolides is strengthened by the fact that bufadienolides were not detected until after metamorphosis, whereas bioassays have found that other *Bufo* larvae are toxic to some invertebrates and unpalatable to some fish (Kruse and Stone 1984, Crossland 1988). An alternative explanation is that *B. boreas* larvae exposed to predator cues could have had a faster physiological burst-speed escape response at the moment of attack than did control larvae. However, a kinematics study of another anuran found no difference in burst speed between tadpoles raised with predators and those raised without predator cues (Van Buskirk and McCollum 2000).

The change in response of the *Aeshna* between the mid-larval bioassay and the late-larval bioassay suggested that predator-treatment *B. boreas* larvae developed more effective defenses against the *Aeshna* than did the control-treatment *B. boreas* during the interval between these two bioassays. If the difference in predator response between the two treatments is due to a defensive chemical that we did not test for, then the change in response of the *Aeshna* indicates an increased toxicity against this predator in the interval between the mid-larval and the late-larval assays. It is unfortunate that we were unable to make a similar comparison between the mid-larval and the late-larval bioassays using *Dytiscus*, but the phenology of this predator made itlogistically prohibitive. It is worth noting that *Aeshna* are chewing predators, whereas the *Abedus* and *Dytiscus* feed by piercing and sucking their prey. Because amphibian toxins are usually concentrated in the skin, it might be expected that *Dytiscus* and *Abedus* would be less susceptible to the toxin than *Aeshna*. However, other sucking predators (Hemiptera: Belostomatidae: *Lethocerus*) have been shown to be vulnerable to toxins in *Bufo* tadpoles (Crossland 1998). This also indicates that an internal toxin other than bufadienolides may be responsible for the induced defense in larvae.

**Costs of induced defenses in other amphibian larvae**

Table 5. ANCOVA of the effects of larval treatment on the time that it took for each adult salamander to catch a cricket placed in its cage, one hour after it swallowed a toad.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>ss</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>5</td>
<td>1986.18</td>
<td>13.49</td>
<td>0.0063</td>
</tr>
<tr>
<td>Error</td>
<td>6</td>
<td>147.28</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>559.21</td>
<td>18.985</td>
<td>0.0073</td>
</tr>
<tr>
<td>Predator mass</td>
<td>1</td>
<td>82.05</td>
<td>2.7855</td>
<td>0.1560</td>
</tr>
<tr>
<td>Toad mass</td>
<td>1</td>
<td>61.169</td>
<td>2.0766</td>
<td>0.2091</td>
</tr>
<tr>
<td>Treatment × toad mass</td>
<td>1</td>
<td>669.08</td>
<td>22.7148</td>
<td>0.0050</td>
</tr>
<tr>
<td>Treatment × predator mass</td>
<td>1</td>
<td>417.120</td>
<td>14.1636</td>
<td>0.0131</td>
</tr>
</tbody>
</table>

**Figure 5.** Total amount (mean ± 1 s.e.) of bufadienolide in postmetamorphic *B. boreas* from the predator treatment vs. the control; the former had significantly higher amounts of bufadienolides (*P* < 0.05).

Only one previous study has tested for postmetamorphic costs to an induced defense. Van Buskirk and Saxer’s (2001) study of Rana ridibunda found no evidence of a postmetamorphic cost. Instead, R. ridibunda from a larval environment with predator cues had improved hopping distances, an apparent benefit. In contrast, B. boreas larvae in our study that were exposed to predator cues as larvae suffered an apparent cost after metamorphosis. Specifically, larvae from the predator-cue treatment were more rapidly eaten by tiger salamanders than were larvae reared in a control environment. Given that B. boreas and other Bufo larvae gather in kin groups and may discriminate kin after metamorphosis (Blaustein et al. 1990, Waldman et al. 1992), predators that take a long time to eat a toad metamorph and to recover from eating a toad metamorph may allow siblings of toads without predator-induced defenses to escape the predator. The more time that it takes for a predator to eat a single toad, the more time is available for that toad’s siblings to escape. In our postmetamorphic bioassays, the tiger salamanders took longer to eat control-treatment B. boreas metamorphs. Additionally, A. tigrinum that had eaten control-treatment B. boreas took significantly longer to catch crickets than those that had eaten predator-treatment B. boreas metamorphs. If we had examined only the costs associated with the larval period, we would have concluded that there was no evidence for a cost of induced defenses. However, our bioassays suggest that individuals that induce a defense do suffer a cost after metamorphosis. Thus, there is incentive to test for postmetamorphic costs in any taxa in which induced defense is expressed in a juvenile or larval stage.

It is important to consider that we only tested one of the many potential terrestrial predators in the postmetamorphic bioassay experiment, and that our interpretation of costs hinges on that bioassay. The defensive efficacy of a particular strategy, whether it be behavioral or chemical defense, is likely to differ among predators (Malcolm 1992). Although ineffective against A. tigrinum and presumably ineffective against garter snakes (Arnold and Wassersug 1978, Chivers et al. 1999), the increased levels of bufadienolide present in metamorphs emerging from predator-cue environments may deter other predators. For example, the concentration of bufadienolides present in toads emerging from larval environments with predator cues may be sufficient to deter other predators such as birds and mammals. The same concentration of chemically similar cardiac glycosides has been shown to elicit a toxic response in some birds and mammals (Malcolm 1991). Additionally, both mammals and birds avoid eating the skin of adult toads (Corn 1993, Brothers 1994). Although experimental studies such as this provide a valuable view of “selection vignettes” of different life stages of an organism, long-term studies assessing life-time fitness are needed to fully understand the consequences of induced defenses.

The difference in handling time between the two treatments in the postmetamorphic bioassay was surprising, given that the predator-treatment B. boreas had significantly higher concentrations of bufadienolides than the control B. boreas. The faster consumption in the postmetamorphic bioassay of B. boreas that had been exposed to predators as larvae would not have been surprising if there had been another obvious difference between the predator and control treatments, such as size or time to metamorphosis. Physiological performance, such as stamina, may have been the important factor in altering the handling time of the predators. From our observations of the encounters with tiger salamanders, B. boreas appeared to struggle for the entire time that they were being consumed. Other amphibians have been shown to defend themselves from predation with physical struggling, such as inflating their bodies, or clinging to the predator (Arnold and Wassersug 1978, Duellman and Trueb 1986, Williams et al. 2000). We hypothesize that the predator-treatment environment caused reduced physiological performance in postmetamorphic B. boreas.

Our study is the first demonstration of postmetamorphic effects of predator-induced larval defenses that are due to treatment effects, rather than by allometric effects of predator-induced plasticity in size and age at metamorphosis. In the only other similar study of which we are aware, Relyea (2001c) found that predator-induced changes in duration of the larval period and size at metamorphosis explained postmetamorphic differences in shape between Rana sylvatica raised with or without predator cues. A dichotomy between allometric effects of size and age at metamorphosis and effects of treatment beyond allometric effects can be found in the literature. Several studies have found that differences in the larval period entirely influence postmetamorphic shape or performance (John-Alder and Morin 1990, Blouin and Loeb 1991). Other studies have found that there are effects of larval condition beyond allometric effects (Goater et al. 1993, Beck and Congdon 2000, Blouin and Brown 2000). The ecological significance of all of these postmetamorphic differences remains uninvestigated.

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January 2003 COSTS OF INDUCED DEFENSE IN WESTERN TOADS


