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Predator-induced changes in the chemical defence of a vertebrate

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Abstract

1. Inducible defences are ubiquitous in the animal kingdom, but little is known about facultative changes in chemical defences in response to predators, especially so in vertebrates.
2. We tested for predator-induced changes in toxin production of larval common toads (*Bufo bufo*), which are known to synthesize bufadienolide compounds.
3. The experiment included larvae originating from three permanent and three temporary ponds reared in the presence or absence of chemical cues of three predators: dragonfly larvae, newts or fish.
4. Tadpoles raised with chemical cues of predation risk produced higher numbers of bufadienolide compounds and larger total bufadienolide quantities than predator-naive conspecifics. Further, the increase in intensity of chemical defence was greatest in response to fish, weakest to newts and intermediate to dragonfly larvae. Tadpoles originating from temporary and permanent ponds did not differ in their baseline toxin content or in the magnitude of their induced chemical responses.
5. These results provide the first compelling evidence for predator-induced changes in chemical defence of a vertebrate that may have evolved to enhance survival under predation risk.

KEYWORDS
among-population variation, antipredator defence, anuran amphibian, local adaptation, phenotypic plasticity, poison

1 | INTRODUCTION

Inducible responses to predators are ubiquitous in the animal kingdom (Tollrian & Harvell, 1999). They evolve because they confer a survival advantage against predators that exceeds whatever fitness costs they may carry. Inducible responses shape ecological patterns and processes and thereby contribute to the diversity, stability and persistence of communities, populations and species (Miner, Sultan, Morgan, Padilla, & Relyea, 2005), and may pave the way for speciation (Pfennig et al., 2010; West-Eberhard, 1989, 2003). Inducible defences can manifest in many forms, including altered behaviour, morphology and life history (Tollrian & Harvell, 1999). However, whether animals are capable of plastically adjusting their chemical defences to the risk of predation, as many plants are (Karban & Baldwin, 1997), is poorly known (Hettyey, Tóth, & Buskirk, 2014).

Chemical defences are found in many animal taxa, and toxins can be effective in deterring predators (Kicklighter, 2012; Toledo & Jared, 1995). Toxicity is known to vary among populations and life stages within species (Bókony et al., 2016; Fordyce, Nice, & Shapiro, 2006; Hayes, Crossland, Hagman, Capon, & Shine, 2009; Kubanek et al., 2002; Ujszegi, Móricz, Krüzselyi, & Hettyey, 2017; Üveges et al., 2017), which may indicate that the physiological machinery of toxin synthesis is flexible. Also, a handful of studies suggest that plastic responses in animal chemical defences may be induced by the appearance of pathogens (Mangoni, Miele, Renda, Barra, & Simmaco, 2001; Miele, Ponti, Boman, Barra, & Simmaco, 1998), competitors (Bókony et al., 2016; Bókony, Üveges, Móricz, & Hettyey, 2018) and even by anthropogenic pollutants (Bókony, Üveges, Verebélyi, Ujhegyi, & Móricz, 2019; Bókony, Zs, Móricz, Krüzselyi, & Hettyey, 2017). Although changes in toxin levels in response to predators are known to exist in some lower invertebrates (e.g. a sponge: Ebel, Brenzinger, Kunze, Gross, & Proksch, 1997; cnidarians: Slattery, Starmer, & Paul, 2001; Thornton & Kerr, 2002), evidence in vertebrates is scarce and controversial.

Benard and Fordyce (2003) and Hagman, Hayes, Capon, and Shine (2009) showed that juvenile toads of two species altered their toxin synthesis after having been raised in the presence of chemical cues indicating predation risk during the larval stage. However, the adaptive significance of these delayed environment-induced changes in toxin production is unclear because predation risk in the terrestrial habitat of juveniles is unlikely to be correlated with that experienced during the aquatic larval stage. Further, Benard and Fordyce (2003) and Üveges et al. (2017, Üveges, Szederkényi,et al.2019) found no effect of predation risk on toxin synthesis in toad larvae. Bucciarelli, Shaffer, Green, and Kats (2017) reported an increase in the quantity of tetrodotoxin in *Taricha torosa* newts resulting from repeated invasive skin sampling. Although they claimed that these changes represented predator-induced responses in chemical defence, this interpretation is uncertain because no natural predators were used in the experiment, and environmental stressors unrelated to predation can also stimulate the production of chemical defences (Bókony et al., 2018, 2017; Mangoni et al., 2001). It is also unclear whether newts, or indeed metazoans in general, are capable of synthesizing tetrodotoxin (Bane, Lehane, Dikshit, O'Riordan, & Furey, 2014; Chau, Kalaitzis, & Neilan, 2011; Magarlamov, Melnikova, & Chernyshev, 2017).

Predation risk can vary among habitats, so that local adaptation can lead to considerable among-population variation in the expression of defences and in the magnitude of its inducible component (Hettyey et al., 2016; Kishida, Trussell, & Nishimura, 2007; Van Buskirk, 2014). The few studies testing the effects of predators on amphibian chemical defences (Benard & Fordyce, 2003; Hagman et al., 2009; Üveges et al., 2017, Üveges, Szederkényi, et al.,2019) used individuals originating from only one or two populations, and may not have been able to detect plastic responses in chemical defences due to accidental choice of populations with low levels of inducibility. This hypothesis is supported by the observation of Bucciarelli et al. (2017) that the changes in the toxin content of repeatedly injured *T. torosa* newts differed between members of the two studied populations.

To perform a comprehensive test of predator-induced changes in the chemical defences of a vertebrate, we conducted an experiment with an anuran amphibian, the common toad (*Bufo bufo* Linnaeus, 1758), which produces bufadienolide toxins (cardiotoxic steroids) starting early in the larval stage (Üveges et al., 2017). We collected freshly laid eggs from three permanent and three temporary ponds, reared the hatched larvae in either the absence or presence of cues of predation risk and assessed their bufadienolide toxin content after 20 days. We simulated predation risk by exposing developing tadpoles to chemical cues originating from injured conspecifics combined with the chemical cues of either dragonfly larvae, newts or fish. Dragonfly larvae and newts are typical top predators of smaller, temporary water bodies, while fishes dominate the predator fauna of permanent ponds and lakes.

We expected to observe elevated bufadienolide content in tadpoles reared in the presence of predator cues. We also predicted that variation in the magnitude of induced changes in toxin production would depend on the danger represented by the predator species and whether it is sensitive to bufadienolides. Of the three predators used in this experiment, fishes are considered the most dangerous to anuran larvae in general, followed by aeshnid dragonfly larvae and newts (Relyea, 2001; Semlitsch, 1993). However, chemical defences of common toad tadpoles appear to be most effective against fish and newts and less effective against invertebrate predators (Gunzburger & Travis, 2005; Henrikson, 1990; Manteifel & Reshetnikov, 2002; Üveges, Szederkényi, et al., 2019). These relationships led us to predict a strong induced chemical defence against fish (dangerous and sensitive), a weaker response to dragonfly larvae (fairly dangerous but not very sensitive) and the weakest response to newts (sensitive but not very dangerous). Further, we expected to find signs of local adaptation to differences in predation risk (Kawecki & Ebert, 2004) in the form of variation among populations in baseline toxin content (i.e. the number and amount of bufadienolides produced when developing in a predator-free environment) and in the intensity of antipredator responses in toxin synthesis. One reason to expect among-population differences is

that continuously high predation risk imposed by fishes in permanent ponds may select for higher baseline toxin production and/or more intense plastic responses than weaker and more variable predation risk in temporary water bodies. Analogous findings have been reported for behavioural and morphological defences (Åbjörnsson, Hansson, & Brönmark, 2004; Herczeg, Turtiainen, & Merilä, 2010; Hettyey et al., 2016; Kishida et al., 2007; Magurran, 1990). However, constantly high predation risk may also purge plasticity in toxin production by selecting for constantly high levels of chemical defences (Crispo, 2007; Pfennig et al., 2010; West-Eberhard, 2003). Also, high baseline levels of toxin production may hinder a further increase in bufadienolide synthesis because of physiological constraints. Therefore, we predicted that compared to tadpoles from temporary ponds, tadpoles from permanent ponds would exhibit higher baseline toxin levels, and perhaps (but not necessarily) also more intense antipredator responses in toxin production.

2 | MATERIALS AND METHODS

2.1 | Experimental procedures

In early spring 2016, we collected 50 eggs from each of ten *B. bufo* egg strings (families) from each of six water bodies located in the Pilis–Visegrádi Mountains, Hungary. Three of these water bodies are permanent ponds inhabited by fish: Apátkúti-tó (P1; 47°46'1.55"N, 18°58'53.11"E), Garancsi-tó (P2; 47°37'25.38"N, 18°48'26.18"E) and Határréti-tó (P3; 47°38'46.90"N, 18°54'31.82"E), while the other three are temporary ponds lacking fish: Jávor-tó (T1; 47°42'50.32"N, 19°1'10.74"E), Békás-tó (T2; 47°34'34.72"N, 18°52'8.06"E) and Szárazfarkas-belső (T3; 47°44'4.12"N, 18°49'7.04"E). We transferred eggs to the experimental station of the Plant Protection Institute (Centre for Agricultural Research, Hungarian Academy of Sciences) in Budapest, where we kept them in the laboratory until hatching. Each family was kept in 0.5 L reconstituted soft water (RSW; 48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4 \times 2\ H_2O$, 61 mg/L $MgSO_4 \times 7H_2O$ and 2 mg/L KCl dissolved in reverse-osmosis filtered tap water and treated with UV). We set room temperature to 20°C during daylight hours and 17°C at night. Lighting was set to a 13.5:10.5-hr light:dark cycle in the beginning; day length was increased by half an hour each week to simulate natural changes in the photoperiod.

The experiment had a 6 × 4 complete factorial design with 10 replicates. Tadpoles from the six source ponds were exposed to four predator treatments (described below), with one tadpole in each predator treatment taken from each of ten replicate families per pond. Two days after the tadpoles hatched, we haphazardly selected four from each family, placed them individually into 2-L rearing containers filled with 0.7 L RSW and assigned them randomly to treatments. Containers were arranged in ten spatial blocks in the laboratory; the 24 containers within each block were assigned positions at random. We changed water twice a week and fed tadpoles on these occasions with a 1:100 mixture of finely ground Spirulina alga powder and slightly boiled, chopped spinach ad libitum.

The four treatments were a predator-free control, chemical cues of adult male smooth newts (*Lissotriton vulgaris*), late-instar emperor dragonfly larvae (*Anax imperator*) and adult European perch (*Perca fluviatilis*). Apparent predation risk was manipulated in the experiment by adding stimulus water to the rearing containers of toad tadpoles twice a week. Stimulus water contained chemical cues originating from the respective predators, their prey (see below) and injured conspecific *B. bufo* tadpoles.

To ensure similar concentrations of chemical cues in the three predator treatments, we adjusted the quantity of water and food provided to predators as follows. We maintained six newts together in a 40-L container (57 × 39 × 28 cm, length × width × height) filled with 8 L RSW. Six late-instar (F-1) dragonfly larvae were kept individually in 2-L containers filled with 1 L RSW and equipped with a plastic perching stick. Six fish were housed together in a 140-L tub (82 × 58 × 30 cm) filled with 105 L aerated RSW (which was later lowered to 95 L; see below). These procedures ensured a constant ratio of predator mass to water volume across all predator species, averaging 1.344 g body mass/L ± 0.021 *SD* at the beginning of the experiment. A few predators were replaced during the experiment because they transformed to the terrestrial form (newts), refused to eat (dragonfly larvae) or spawned (fish). We took care to use similar-sized individuals and adjusted water levels if necessary to ensure a constant concentration of cues. Five times a week we fed predators with one agile frog (*Rana dalmatina*) tadpole (a preferred prey of all three predators) and ca. five *Tubifex* worms for every 2 L of RSW. Thus, the group of six fish received a total of 52 tadpoles on each feeding occasion (47 after readjustment of the water volume), the six newts received four tadpoles, and each dragonfly larva received one tadpole on every other feeding occasion. We did not weigh the tadpoles used as food, but chose similar-sized individuals at each feeding. *Rana dalmatina* tadpoles were used to guarantee that predators consumed prey and generated equal amounts of prey-borne cues in all treatments. This would not have been feasible if we had fed predators with toad tadpoles, because smooth newts are reluctant to feed on *Bufo* tadpoles and perch consume *Bufo* but avoid them if possible, while *Anax* larvae readily feed solely on *Bufo* (Henrikson, 1990; Manteifel & Reshetnikov, 2002; Üveges, Szederkényi, et al., 2019).

Toad tadpoles in the predator treatments also received chemical cues originating from injured and killed conspecifics. We homogenized 138.5 ± 2.4 mg (mean ± *SD*) common toad tadpoles using a blender in 150 ml water and added the homogenate to 2 L water taken from the housing container(s) of each predator species. Five times a week we pipetted 20 ml freshly prepared stimulus water into the rearing containers of *Bufo* tadpoles assigned to the respective predator treatments. Simultaneously, we added 20 ml RSW into rearing containers of tadpoles in the control treatment. Tadpoles homogenized using a blender perish almost instantly, and therefore may not produce or release all types of chemical cues in the same quantity as during a natural predation event (Fraker et al., 2009), but similar methods have been used before and result in strong induced responses in tadpoles (Hagman et al., 2009; Hettyey et al.,

2015; Schoeppner & Relyea, 2005). The procedure described above resulted in 8.3 mg conspecific tadpole tissue per L per week in the rearing containers of focal tadpoles. Similar and also lower concentrations of chemical cues of predation risk have been shown to induce plastic responses in amphibian larvae (Hettyey et al., 2015; McCoy, Touchon, Landberg, Warkentin, & Vonesh, 2012; Van Buskirk & Arioli, 2002). After preparation of stimulus water, we filled the containers of predators with RSW to the original level.

To be able to assess treatment effects on toxin content of toad tadpoles, we preserved all 240 individuals in HPLC-grade absolute methanol 20 days after the start of the experiment, when tadpoles were at developmental stage 35 (Gosner, 1960). We chose this age to give tadpoles enough time to respond to treatments, to grow large enough to enable the quantification of toxin content, and because other work suggests that bufadienolide content of *Bufo* tadpoles is highest when they are about three weeks old (Ujszegi et al., 2017; Üveges et al., 2017). All tadpoles survived to the end of the experiment.

2.2 | Analysis of toxin content

We used high-performance liquid chromatography with diode-array detection and mass spectrometry (HPLC-DAD-MS, Model LC-MS-2020; Shimadzu) to identify and quantify bufadienolide compounds. Tadpoles were homogenized with an IKA S12N-7S dispersing tool attached to a VWR VDI 12 homogenizer. We then dried samples *in vacuo* at 45°C using a Büchi Rotavapor R-134 rotary evaporator and measured dry mass to the nearest 0.1 mg using an analytical balance. Samples were re-dissolved in 1 ml HPLC-grade absolute methanol, facilitated by brief exposure to ultrasound in a Tesla UC005AJ1 bath sonicator. We filtered samples using FilterBio nylon syringe filters (pore size: 0.22 μm). We identified compounds as bufadienolides by inspecting the UV (Benard & Fordyce, 2003; Bókony et al., 2016; Hagman et al., 2009) and HRMS/MS spectra of peaks using a QTOF Premier mass spectrometer (Waters Corporation, Manchester, UK) in positive electrospray mode, and by comparing them to the following commercially acquired bufadienolides as standards: bufalin, bufotalin, resibufogenin, gamabufotalin, areno- and telocinobufagin (Biopurify Phytochemicals, Chengdu, China), cinobufagin (Chembest, Shanghai, China), cinobufotalin (Quality Phytochemicals) and digitoxigenin (Santa Cruz Biotechnology). Identification of compounds present in low quantities as bufadienolides was further aided by the chemical analysis of a pooled sample obtained from 49 juvenile *Bufo* by massaging their parotoid glands.

The HPLC-MS system (Model LC-MS-2020; Shimadzu) was equipped with a binary gradient solvent pump, a vacuum degasser, a thermostated autosampler, a column oven, a photodiode detector and a single-quadrupole mass analyser with electrospray ionization (ESI/MS). We injected 10 μl of each sample at 35°C on a Kinetex C18 2.6-μm column (100 mm × 3 mm i.d.; Phenomenex) protected by a C18 guard column (4 mm × 3 mm i.d.; Phenomenex). Eluent A was 5% aqueous acetonitrile with 0.05% formic acid, and eluent B was acetonitrile with 0.05% formic acid. The flow rate was 0.6 ml/min, and the gradient was as follows: 0–2 min: 10%–20% B; 2–15 min: 20%–32% B; 15–21 min: 32%–60% B; 21–21.5 min: 60%–100% B; 21.5–26 min: 100% B; and 26–30 min: 10% B. We set ESI conditions as follows: interface temperature: 350°C; desolvation line (DL) temperature: 250°C; heat block temperature: 400°C; drying N_2 gas flow: 15 L/min; nebulizer N_2 gas flow: 1.5 L/min; and positive ionization mode. We recorded full-scan spectra in the range of 350–800 m/*z* and also performed selected-ion monitoring (SIM) acquisition detecting the base peak of bufadienolides we previously found in common toads (Bókony et al., 2016; Üveges et al., 2017). We acquired and processed data using the software LabSolutions 5.42v (Shimadzu Corp.).

2.3 | Statistical analysis

One sample was lost during preparation for HPLC-DAD-MS analysis, resulting in a sample size of 239 tadpoles. We determined the number of bufadienolide compounds (NBC) for each tadpole by assuming that a compound was present if the signal-to-noise ratio (S/N) of its peak, calculated from appropriate SIM chromatogram by the LabSolutions software, was at least three. We estimated the quantity of each bufadienolide compound from the area of its chromatogram peak using the calibration curve of the bufotalin standard, and we obtained estimates of total bufadienolide quantity (TBQ) for each tadpole by summing up these values. This approach yields only a rough estimate of TBQ, but due to the unavailability of standards for the majority of bufadienolides, there is currently no better alternative for toxin quantification. This method has been successfully applied in similar studies (Benard & Fordyce, 2003; Bókony et al., 2016, 2018, 2019, 2017; Hagman et al., 2009; Tóth, Kurali, Móricz, & Hettyey, 2019; Üveges et al., 2017). We calculated mass-corrected total bufadienolide quantities (mcTBQ) by dividing TBQ values by tadpole dry mass. We calculated mcTBQ to estimate individual investment (i.e. proportion of resources allocated to toxin synthesis), while TBQ is more likely to be relevant for the actual outcome of predatory interactions.

We investigated if predator treatments affected growth and development rates by analysing variation in tadpole body mass (dry mass at toxin sampling) using a linear mixed-effects model, entering predator treatment as a fixed factor and family nested within population crossed with block as random factors. Because developmental stage (Gosner, 1960) in our sample had only a few discrete values (79% of individuals were in stage 35 or 36), we used Mann–Whitney U tests to compare developmental stage of tadpoles in the control treatment to those in each predator treatment. We corrected *p*-values for the number of comparisons by applying the false discovery rate (FDR) method (Benjamini & Hochberg, 1995). There was a reduced sample size for developmental stage because we assessed this trait in only a subset of individuals ($N = 48$, i.e. two replicates for each combination of predator treatment by population). Tadpoles showed little variation in stage (range: 33–37), and in a previous experiment where we used tadpoles in very similar developmental stages (range: 32–35), we

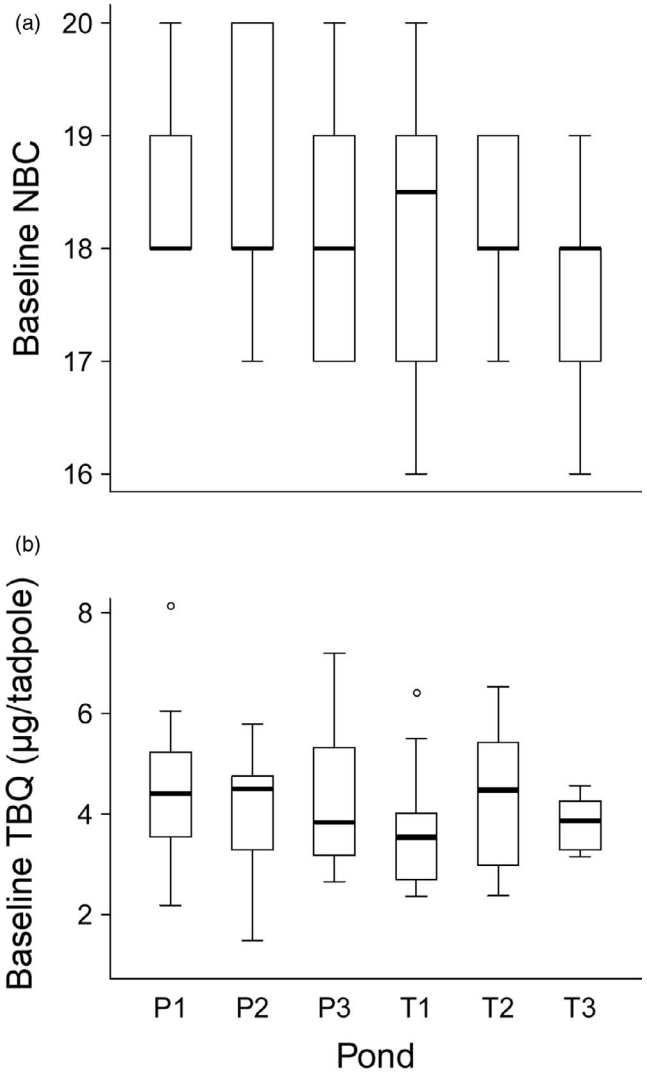

FIGURE 1 The number of bufadienolide compounds (NBC, upper panel) and total bufadienolide quantity (TBQ, lower panel) in control tadpoles reared in the absence of cues of predation risk separated by their population of origin. Thick horizontal lines represent medians, boxes represent the interquartile ranges, and whiskers extend to the upper and lower quartile ± 1.5 × interquartile range; open circles represent extreme data points. Numbering of permanent (P) and temporary (T) ponds of origin corresponds with that in the 'Methods' section

found no correlation between developmental stage and toxin content (Bókony et al., 2017).

We ran three linear mixed-effects models, one for NBC, one for TBQ and one for mcTBQ, entering predator treatment and population and their interaction as fixed factors, and block crossed with family as random factors. From each model, we calculated the following pre-planned comparisons (linear contrasts; for R scripts, see supplementary material). First, we assessed among-population differences in baseline toxin production, that is in the absence of predator cues, by comparing the control group's estimated confidence intervals between the six ponds. We also compared baseline toxin levels between permanent and temporary ponds by estimating the difference between the mean of the three permanent ponds and the mean of the three temporary ponds in the absence of predator cues.

Second, to test for predator-induced responses in toxin production, we first estimated among-treatment differences irrespective of among-population differences, calculating the overall difference between the control group (mean of six populations) and each predator treatment (mean of six populations). Next, to assess among-population differences in antipredator responses, we calculated differences in toxin production between the control and each predator treatment within each population. Finally, we calculated the difference between permanent and temporary ponds in the response to each predator (i.e. difference between the control and the respective predator treatment), as a linear contrast of the within-population contrasts (i.e. comparing the average response of the three permanent-pond populations to the average response of the three temporary-pond populations). In each step, we corrected *p*-values for the number of comparisons by the FDR method.

Throughout the statistical analyses on toxin content of tadpoles, we used the approach of planned comparisons (Ruxton & Beauchamp, 2008), in which we first estimated the mean of each population (i.e. mean baseline toxin content, or mean response in toxin content in response to each predator) in a linear model and then estimated the effect of pond permanence as the difference between the mean of the three permanent ponds and the mean of the three temporary ponds. This approach has two main advantages over using pond permanence as fixed effect and pond as random effect. First, because ponds are nested within the two permanence categories, and there were only three ponds of each category, a mixed model with pond as random effect would have low power for testing the fixed effect of pond permanence. Second, estimating the variance component due to a random effect is reliable only when the number of levels is large (Bolker et al., 2009), so we could not evaluate variance among populations if pond were included as a random effect. Therefore, pond was a fixed effect as detailed below.

We confirmed that our data fit the assumptions of analyses by inspecting residual plots. Mixed-effects models were fitted using the 'lmer' function of the 'lme4' package (Bates, Mächler, Bolker, & Walker, 2015) in R v. 3.4.0 (R Core Team, 2017). Satterthwaite approximation was used to calculate degrees of freedom. For calculating linear contrasts, we used the 'lsmeans' package (Lenth, 2016). We report least-squares means with standard errors (*SE*s) and with 84% confidence intervals (CIs) to facilitate comparisons between populations, because the lack of overlap between two 84% CIs indicates a significant difference, that is is equivalent to a 95% CI around the difference not including zero (Julious, 2004).

3 | RESULTS

3.1 | Treatment effects on body mass and development

At the termination of the experiment, body mass of tadpoles raised in the presence of chemical cues from dragonfly larvae and fish

TABLE 1 Estimates of linear contrasts and their *p*-values corrected for false discovery rate, comparing the number of bufadienolide compounds (NBC) and total bufadienolide quantity (TBQ) between treatments. Significant differences are highlighted in bold

Trait	Contrasts	Estimate ± *SE*	*df*	84% CI	*t*	*p*
NBC	Control versus newt	**0.889 ± 0.139**	**156.60**	0.693, 1.086	**6.39**	**<.001**
	Control versus dragonfly	**1.100 ± 0.138**	**156.01**	0.904, 1.296	**7.94**	**<.001**
	Control versus fish	**1.367 ± 0.138**	**156.01**	1.171, 1.562	**9.87**	**<.001**
	Newt versus dragonfly	0.211 ± 0.139	156.60	0.014, 0.407	1.51	.132
	Newt versus fish	**0.477 ± 0.139**	**156.60**	0.281, 0.674	**3.43**	**.001**
	Dragonfly versus fish	0.267 ± 0.138	156.01	0.071, 0.462	1.93	.067
TBQ	Control versus newt	**494.13 ± 191.93**	**123.56**	222.81, 765.44	**2.57**	**.014**
	Control versus dragonfly	**541.89 ± 190.81**	**123.29**	272.16, 811.61	**2.84**	**.008**
	Control versus fish	**1,084.36 ± 190.81**	**123.29**	814.63, 1,354.09	**5.68**	**<.001**
	Newt versus dragonfly	47.76 ± 191.93	123.56	−223.55, 319.07	0.25	.804
	Newt versus fish	**590.23 ± 191.93**	**123.56**	318.92, 861.55	**3.08**	**.008**
	Dragonfly versus fish	**542.47 ± 190.81**	**123.29**	272.75, 812.20	**2.84**	**.008**

was significantly lower than in predator-naïve tadpoles, while the mass of tadpoles exposed to cues from newts did not differ from that of controls (Table S1, Figure S1). Tadpoles exposed to cues from newts tended to be more developed than control tadpoles (Mann–Whitney *U* test; $W = 40.5$, $p = .07$; Figure S1), whereas those raised in the presence of cues from fish were slightly less developed than controls ($W = 104$, $p = .07$; Figure S1), and tadpoles exposed to dragonfly cues did not differ in developmental stage from controls ($W = 82.5$, $p = .52$; Figure S1).

3.2 | Baseline toxin content

The analysis on control tadpoles reared in the absence of cues of predation risk revealed no significant variation among populations either in NBC or in TBQ (Table S2, Figure 1). Linear contrasts indicated that baseline NBC and TBQ did not differ between tadpoles originating from permanent and temporary ponds (difference in NBC: 0.37 ± 0.21 bufadienolide compounds, $t_{191.3} = 1.71$, $p = .09$; in TBQ: 293.06 ± 368.21 ng bufadienolides per tadpole, $t_{125.9} = 0.8$, $p = .43$; in mcTBQ: 0.05 ± 0.05 ng bufadienolides per mg tadpole mass, $t_{194.9} = 0.95$, $p = .34$).

3.3 | Plasticity in toxin production

Tadpoles exposed to predators responded with the production of increased numbers of bufadienolide compounds (Table 1; Figure 2). Linear contrasts revealed that NBC was significantly lower in predator-naive tadpoles (18.18 ± 0.13 compounds; mean ± *SE*) than in tadpoles exposed to cues of any species of predator and that the response was strongest to fish (19.55 ± 0.07), intermediate to dragonflies (19.28 ± 0.11) and weakest to newts (19.07 ± 0.12; Table 1, Figures 2 and 3). We detected significant variation among tadpoles according to population of origin in the intensity of predator-induced changes in NBC as indicated by non-overlapping 84% confidence intervals (Table S3, Figure S2). However, these differences were not attributable to pond permanence: tadpoles from both types of ponds produced significantly higher numbers of bufadienolide compounds in response to each of the three predator species, but this response did not differ between permanent and temporary ponds (Table 2, Figure 3).

Total bufadienolide quantity also responded to the predator treatments (Table 1, Figure 2). Tadpoles in the control treatment produced the lowest TBQ (4,155.72 ± 164.66 ng per tadpole; mean ± *SE*) and those reared in the presence of cues from fish the highest (5,240.08 ± 180.57), whereas tadpoles exposed to cues of newts and dragonflies contained intermediate toxin levels (newts: 4,658.7 ± 215.6; dragonflies: 4,697.6 ± 173.41; Table 1, Figures 2 and 3). Linear contrasts indicated that predator-naive tadpoles had significantly lower TBQ than tadpoles in any other treatment (Table 1, Figure 2). We did not detect significant among-population variation in the intensity of predator-induced changes in TBQ, except for a slight difference in response to fish cues between ponds P3 and T3 (Figure S2). When analysing antipredator responses in TBQ by pond permanence type, we found that tadpoles originating from temporary ponds responded to dragonflies with increased toxin production, and so did tadpoles originating from both types of water bodies exposed to chemical cues of fish (Table 2, Figure S2). On the other hand, the response to newts in either type of water body and the response to dragonflies in permanent ponds were marginally non-significant after FDR adjustment. However, linear contrasts did not reveal significant differences in the magnitude of responses between tadpoles originating from temporary and permanent ponds (Table 2; Figure 3).

We obtained qualitatively similar results when analysing variation in mass-corrected total bufadienolide quantity (mcTBQ); in some ponds, these responses were even stronger than the responses observed in TBQ (see Tables S2–S5; Figure S3).

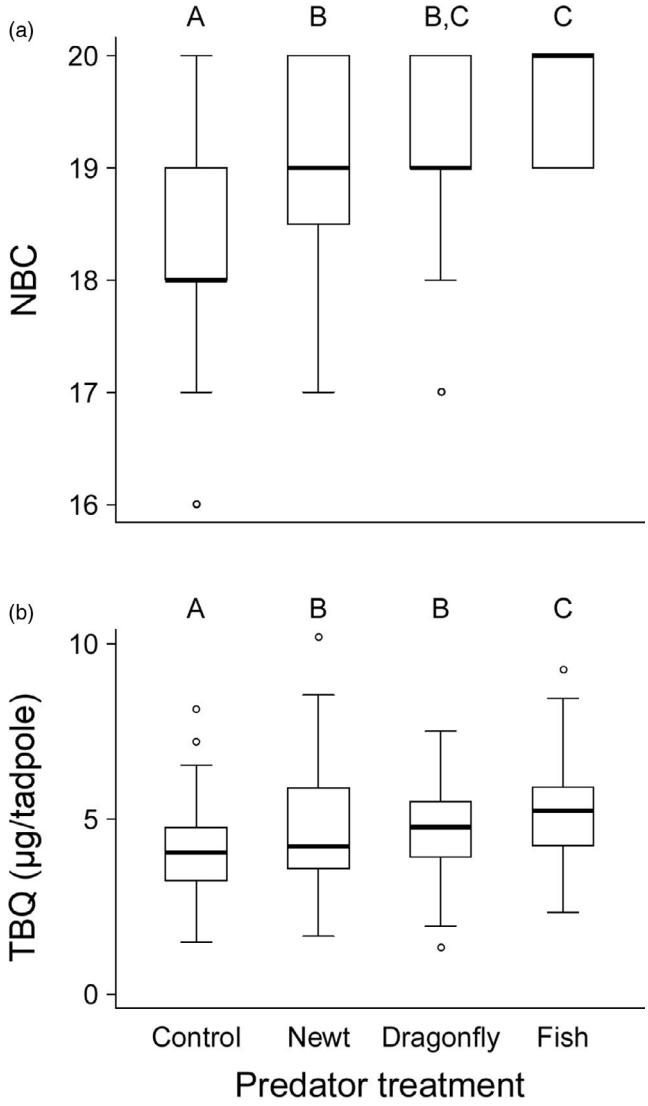

FIGURE 2 The number of bufadienolide compounds (NBC, upper panel) and total bufadienolide quantity (TBQ, lower panel) in treatments differing in predation risk. Thick horizontal lines depict medians, boxes depict the interquartile ranges, and whiskers extend to the upper and lower quartile ± 1.5 × interquartile range; open circles represent extreme data points. Letters above boxplots indicate homogeneous subsets according to pairwise comparisons based on linear contrasts corrected for false discovery rate

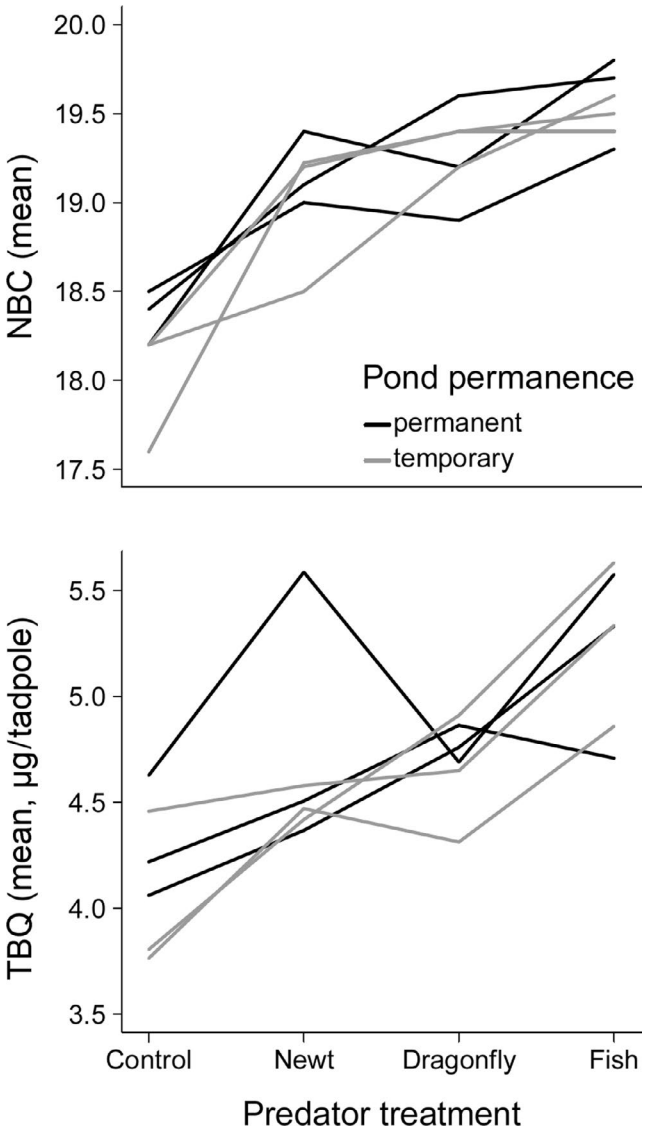

FIGURE 3 Mean values of each population for the number of bufadienolide compounds (NBC, upper panel) and total bufadienolide quantity (TBQ, lower panel) in treatments differing in predation risk. Each line represents one population. The increase in bufadienolide production induced by predators was similar in tadpoles from permanent and temporary ponds. For standard errors of the mean values, see Figure S4

4 | DISCUSSION

Our results demonstrate predator-induced changes in the chemical defence of *Bufo bufo* larvae. Tadpoles reared in the presence of chemical cues of predation risk produced a larger number of bufadienolide compounds and higher total bufadienolide quantity than did tadpoles that developed in a predator-free environment. There was a detectable increase in toxin production in the presence of three very different predator taxa. Furthermore, the strength of induced responses depended on the species of predator present in the larval environment, with fish causing the greatest response. Although plasticity in toxin production did vary significantly among populations, neither baseline toxin content in the absence of predators nor the magnitude of predator-induced responses differed significantly between tadpoles originating from permanent and temporary ponds.

Our study is the first to deliver clear evidence for predator-induced changes in the chemical defence of a vertebrate that can be interpreted as adaptive phenotypic plasticity. Although it has been known for two decades that invertebrates can plastically adjust their toxin production to the presence of predators in ways that enhance survival (e.g. Ebel et al., 1997; Slattery et al., 2001; Thornton & Kerr, 2002), similar reports for vertebrates have so far provided only circumstantial evidence (Benard & Fordyce, 2003; Bucciarelli et al., 2017; Hagman et al., 2009). Results of the present study also

TABLE 2 Treatment effects on the number of bufadienolide compounds (NBC) and total bufadienolide quantity (TBQ) in tadpole populations originating from temporary (T) and permanent (P) ponds. Estimates of linear contrasts compare tadpoles reared in the control treatment to those exposed to chemical cues of newts, dragonfly larvae or fish, within each population type, that is permanent (P) or temporary (T) ponds. *p*-values were corrected for false discovery rate. We also present comparisons of the effects of predator treatment (i.e. the difference between control and predator treatment) between permanent and temporary ponds (P vs. T) based on linear contrasts of the within-population contrasts. Significant differences are highlighted in bold

Trait	Contrasts	Pond type	Estimate ± *SE*	*df*	84% CI	t	*p*
NBC	Control versus newt	**T**	**0.979 ± 0.196**	**157.17**	**0.699, 1.258**	**4.95**	**<.001**
		P	**0.800 ± 0.196**	**156.01**	**0.524, 1.076**	**4.09**	**<.001**
		P versus T	−0.179 ± 0.278	156.60	−0.572, 0.214	−0.64	.522
	Control versus dragonfly	**T**	**1.333 ± 0.196**	**156.01**	**1.057, 1.610**	**6.81**	**<.001**
		P	**0.867 ± 0.196**	**156.01**	**0.590, 1.143**	**4.43**	**<.001**
		P versus T	−0.467 ± 0.277	156.01	−0.858, −0.076	−1.69	.094
	Control versus fish	**T**	**1.500 ± 0.196**	**156.01**	**1.224, 1.776**	**7.66**	**<.001**
		P	**1.233 ± 0.196**	**156.01**	**0.957, 1.510**	**6.30**	**<.001**
		P versus T	−0.267 ± 0.277	156.01	−0.658, 0.124	−0.96	.337
TBQ	Control versus newt	T	470.4 ± 273.0	123.82	84.4, 856.3	1.72	.087
		P	517.9 ± 269.8	123.29	136.4, 899.3	1.92	.087
		P versus T	47.5 ± 383.9	123.56	−495.1, 590.1	0.12	.902
	Control versus dragonfly	**T**	**614.8 ± 269.8**	**123.29**	**233.3, 996.2**	**2.28**	**.049**
		P	469.0 ± 269.8	123.29	87.5, 850.4	1.74	.085
		P versus T	−145.8 ± 381.6	123.29	−658.3, 393.6	−0.38	.703
	Control versus fish	**T**	**1,265.9 ± 269.8**	**123.29**	**884.5, 1647.4**	**4.69**	**<.001**
		P	**902.8 ± 269.8**	**123.29**	**521.3, 1,284.3**	**3.35**	**.001**
		P versus T	−363.1 ± 381.6	123.29	−902.6, 176.3	−0.95	.343

indicate that induced changes in chemical defences can vary depending on the predator species present, much as they do for other defensive traits (Hettyey, Vincze, Zsarnóczai, Hoi, & Laurila, 2011; Kishida & Nishimura, 2005; Relyea, 2001; Sih, 1986; Van Buskirk, 2001). The question arises whether the responses to the different predators could be predicted based on the information available on their relationship with prey. The level of defence should depend on its benefits and costs. Fishes are the most voracious predators of tadpoles in general, followed by dragonfly larvae and newts. At the same time, vertebrate predators are more sensitive to the toxins produced by toad tadpoles than invertebrate predators (Gunzburger & Travis, 2005; Henrikson, 1990; Manteifel & Reshetnikov, 2002; Üveges, Szederkényi, et al., 2019). Thus, the highest benefit of toxin production is expected when the predator is potentially dangerous and highly voracious but also sensitive to the toxins (Üveges, Szederkényi, et al., 2019). Finally, costs of enhanced bufadienolide production are expected to be substantial (Hettyey et al., 2014), and this was recently demonstrated in subadult and adult toads (Blennerhassett, Bell-Anderson, Shine, & Brown, 2019), although clear evidence for such costs in tadpoles has remained elusive (Benard & Fordyce, 2003; Hagman et al., 2009; Kurali, Pásztor, Hettyey, & Tóth, 2016; Üveges et al., 2017). Consequently, our observation that tadpoles produced the highest number and quantity of bufadienolide compounds in the presence of fish, the lowest in response to adult newts and intermediate levels in response to dragonfly larvae corresponds well to what is expected of an adaptive inducible defence (see also Üveges, Szederkényi, et al., 2019).

It is theoretically possible that predators could influence toxin production indirectly by affecting tadpole body size and development rate. Indeed, *B. bufo* larvae modify their growth and development rates under predation risk (e.g. Lardner, 2000; Laurila, Kujasalo, & Ranta, 1998; Van Buskirk, 2000), and toxin content is known to change during development (Ujszegi et al., 2017; Üveges et al., 2017). However, the details of our findings cannot be explained by simple developmental scaling of toxin production. Both NBC and TBQ consistently increased in response to all tested predators, while development rate and growth responded to different predators in different directions. At the same time, we observed that treatments inducing the largest increase in toxin production also caused the greatest decline in tadpole mass. While this may suggest that increasing toxin production is costly, that interpretation would be premature because the experiment was not designed to properly separate treatment-induced changes in individual traits from trade-offs among these traits.

Our finding that predation risk can induce changes in the toxin production of common toad tadpoles contradicts the results of two previous studies with this species (Üveges et al., 2017, Üveges, Szederkényi, et al.,2019). There are three possible explanations for this discrepancy. First, earlier experiments included tadpoles from only one population each, which may have by chance exhibited little plasticity in chemical defence. Indeed, populations can vary in their

responses to environmental cues (Åbjörnsson et al., 2004; Crispo, 2007; Hettyey et al., 2016; Magurran, 1990; Pfennig et al., 2010; West-Eberhard, 2003), and the present study shows that this is also true for the strength of antipredator responses in toxin production. Second, the sample size per treatment was about five times higher in the present experiment than in the previous studies, and this may have resulted in a decisive improvement in statistical power. Finally, previous experiments raised tadpoles in groups (three tadpoles in 1.5 L and 60 tadpoles in 130 L), whereas in the present study we reared tadpoles individually. It is known that the presence of conspecifics in the environment can affect the expression of inducible defences due to prey risk assessment taking into account risk dilution and group vigilance (Peacor, 2003; Tollrian, Duggen, Weiss, Laforsch, & Kopp, 2015; Van Buskirk, Ferrari, Kueng, Näpflin, & Ritter, 2011). Moreover, we recently discovered that *B. bufo* tadpoles adjust their toxin production to the density of conspecifics even in the absence of predators (Bókony et al., 2018), and the toxin content induced by high densities may be high enough for effective protection from various predators (Üveges, Szederkényi et al., 2019). All three of these explanations seem possible, and together they suggest that the contradiction between this study and previous findings may have been caused by chance effects and differences in methodology.

We found no evidence that chemical defences of toad tadpole populations are locally adapted to pond permanence. Although populations varied in their induced antipredator responses, those originating from temporary or permanent ponds did not show the strongest responses to predator taxa that predominate in their pond type. The hypothesis of local adaptation predicts that tadpoles from permanent ponds should show the greatest response to fish, whereas tadpoles from temporary ponds should respond more strongly to dragonflies or newts. These predictions were not upheld (Figure S2). For other kinds of inducible defence—for example involving behaviour, morphology and life history—populations exposed to continuously high predation risk sometimes exhibit more defended phenotypes and more intense antipredator responses than populations originating from low-risk habitats (Åbjörnsson et al., 2004; Herczeg et al., 2010; Hettyey et al., 2016; Kishida et al., 2007; Magurran, 1990). The absence of local adaptation in our study could reflect the swamping effect of gene flow (Blanquart, Gandon, & Nuismer, 2012; Kawecki & Ebert, 2004; Yeaman & Otto, 2011) between permanent ponds and temporary puddles, which are frequently situated immediately adjacent to one another in our study area. Also, selection favouring adaptation to either type of pond could be weakened by microhabitat heterogeneity within ponds. For example, permanent wetlands with fish often have shallow areas that are inaccessible to fish, and these provide safe refugia for tadpoles. Finally, chemical defences of toads are in general more effective against vertebrate than invertebrate predators (Gunzburger & Travis, 2005; Henrikson, 1990; Manteifel & Reshetnikov, 2002), and even low quantities of bufadienolides can provide efficient defences against fishes (Üveges, Szederkényi, et al., 2019). Consequently, ecological factors other than fish presence, such as the density of other predators or conspecifics, may be more important in determining the strength of selection on chemical defences and on plasticity therein.

In conclusion, this study provides clear evidence for inducible responses to predators in chemical defences of a vertebrate. Four arguments suggest that these responses could reflect an adaptive outcome of natural selection imposed by predators: (a) the inducible changes in toxin synthesis occurred in the same environment in which animals encountered cues indicating risk, (b) the observed changes were induced by predators that coexist with *B. bufo* tadpoles in natural populations, (c) the direction of the response (i.e. an increase in both NBC and TBQ induced by predators) indicates that the response is likely to be beneficial to a tadpole under predation risk, and (d) the magnitude of the response varied among predators as predicted by the theory of adaptive phenotypic plasticity; that is, the strongest response was elicited when it was most beneficial because the predator species was potentially highly dangerous and at the same time also highly sensitive to the toxins. Nonetheless, it remains an open question whether antipredator responses in toxin synthesis of toad tadpoles are indeed adaptive, and how frequently predator-induced changes in chemical defences occur in the animal kingdom.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

A.H., B.Ü., J.V.B., R.J.C., Z.T. and V.B. conceived the study; A.H., B.Ü. and V.B. designed the study and conducted statistical analyses; B.Ü. conducted the experiment and prepared samples for chemical analysis; Á.M.M. and L.D. analysed toxin samples; and A.H., B.Ü., Á.M.M., J.V.B. and V.B. wrote the manuscript, and all other authors contributed to its final version.

DATA AVAILABILITY STATEMENT

The dataset analysed in the current study is available on figshare: https://doi.org/10.6084/m9.figshare.8256620.v1 (Üveges, Hettyey, et al. 2019)

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REFERENCES

Åbjörnsson, K., Hansson, L.-A., & Brönmark, C. (2004). Responses of prey from habitats with different predator regimes: Local adaptation and heritability. *Ecology*, *85*, 1859–1866. https://doi.org/10.1890/03-0074

Bane, V., Lehane, M., Dikshit, M., O'Riordan, A., & Furey, A. (2014). Tetrodotoxin: Chemistry, toxicity, source, distribution and detection. *Toxins*, *6*, 693–755. https://doi.org/10.3390/toxins6020693

Bates, D., Mächler, M., Bolker, B. M., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*, 1–48.

Benard, M. F., & Fordyce, J. A. (2003). Are induced defenses costly? Consequences of predator-induced defenses in western toads, *Bufo boreas*. *Ecology*, *84*, 68–78. https://doi.org/10.1890/0012-9658(2003)084[0068:AIDCCO]2.0.CO;2

Benjamini, Y., & Hochberg, Y. (1995). Controlling for false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*, *67*, 289–300.

Blanquart, F., Gandon, S., & Nuismer, S. (2012). The effects of migration and drift on local adaptation to a heterogeneous environment. *Journal of Evolutionary Biology*, *25*, 1351–1363. https://doi.org/10.1111/j.1420-9101.2012.02524.x

Blennerhassett, R. A., Bell-Anderson, K., Shine, R., & Brown, G. P. (2019). The cost of chemical defence: The impact of toxin depletion on growth and behaviour of cane toads (*Rhinella marina*). *Proceedings of the Royal Society B: Biological Sciences*, *286*, 20190867.

Bókony, V., Móricz, Á. M., Zs, T., Gál, Z., Kurali, A., Zs, M., … Hettyey, A. (2016). Variation in chemical defense among natural populations of common toad, *Bufo bufo*, tadpoles: The role of environmental factors. *Journal of Chemical Ecology*, *42*, 329–338. https://doi.org/10.1007/s10886-016-0690-2

Bókony, V., Üveges, B., Móricz, Á. M., & Hettyey, A. (2018). Competition induces increased toxin production in toad larvae without allelopathic effects on heterospecific tadpoles. *Functional Ecology*, *32*, 667–675. https://doi.org/10.1111/1365-2435.12994

Bókony, V., Üveges, B., Verebélyi, V., Ujhegyi, N., & Móricz, Á. M. (2019). Toads phenotypically adjust their chemical defences to anthropogenic habitat change. *Scientific Reports*, *9*, 3163. https://doi.org/10.1038/s41598-019-39587-3

Bókony, V., Zs, M., Móricz, Á. M., Krüzselyi, D., & Hettyey, A. (2017). Chronic exposure to a glyphosate-based herbicide makes toad larvae more toxic. *Proceedings of the Royal Society B: Biological Sciences*, *284*, 20170493. https://doi.org/10.1098/rspb.2017.0493

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H., & White, J. S. S. (2009). Generalized linear mixed models: A practical guide for ecology and evolution. *Trends in Ecology & Evolution*, *24*, 127–135. https://doi.org/10.1016/j.tree.2008.10.008

Bucciarelli, G. M., Shaffer, H. B., Green, D. B., & Kats, L. B. (2017). An amphibian chemical defense phenotype is inducible across life history stages. *Scientific Reports*, *7*, 8185. https://doi.org/10.1038/s41598-017-08154-z

Chau, R., Kalaitzis, J. A., & Neilan, B. A. (2011). On the origins and biosynthesis of tetrodotoxin. *Aquatic Toxicology*, *104*, 61–72. https://doi.org/10.1016/j.aquatox.2011.04.001

Core Team, R. (2017). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/.

Crispo, E. (2007). The Baldwin effect and genetic assimilation: Revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution*, *61*, 2469–2479. https://doi.org/10.1111/j.1558-5646.2007.00203.x

Ebel, R., Brenzinger, M., Kunze, A., Gross, H. J., & Proksch, P. (1997). Wound activation of protoxins in marine sponge *Aplysina aerophoba*. *Journal of Chemical Ecology*, *23*, 1451–1462. https://doi.org/10.1023/B:JOEC.0000006475.10310.3a

Fordyce, J. A., Nice, C. C., & Shapiro, A. M. (2006). A novel trade-off of insect diapause affecting a sequestered chemical defense. *Oecologia*, *149*, 101–106. https://doi.org/10.1007/s00442-006-0428-x

Fraker, M. E., Hu, F., Cuddapah, V., McCollum, S. A., Relyea, R. A., Hempel, J., & Denver, R. J. (2009). Characterization of an alarm pheromone secreted by amphibian tadpoles that induces behavioral inhibition and suppression of the neuroendocrine stress axis. *Hormones and Behavior*, *55*, 520–529. https://doi.org/10.1016/j.yhbeh.2009.01.007

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, *16*, 183–190.

Gunzburger, M. S., & Travis, J. (2005). Critical literature review of the evidence for unpalatability of amphibian eggs and larvae. *Journal of Herpetology*, *39*, 547–571. https://doi.org/10.1670/1-05A.1

Hagman, M., Hayes, R. A., Capon, R. J., & Shine, R. (2009). Alarm cues experienced by cane toad tadpoles affect post-metamorphic morphology and chemical defences. *Functional Ecology*, *23*, 126–132. https://doi.org/10.1111/j.1365-2435.2008.01470.x

Hayes, R. A., Crossland, M. R., Hagman, M., Capon, R. J., & Shine, R. (2009). Ontogenetic variation in the chemical defences of cane toads (*Bufo marinus*): Toxin profiles and effects on predators. *Journal of Chemical Ecology*, *35*, 391–399.

Henrikson, B.-I. (1990). Predation on amphibian eggs and tadpoles by common predators in acidified lakes. *Holarctic Ecology*, *13*, 201–206. https://doi.org/10.1111/j.1600-0587.1990.tb00609.x

Herczeg, G., Turtiainen, M., & Merilä, J. (2010). Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): Signatures of parallel evolution. *Biological Journal of the Linnean Society*, *101*, 403–416. https://doi.org/10.1111/j.1095-8312.2010.01518.x

Hettyey, A., Thonhauser, K. E., Bókony, V., Penn, D. J., Hoi, H., & Griggio, M. (2016). Naive tadpoles do not recognize recent invasive predatory fishes as dangerous. *Ecology*, *97*, 2975–2985.

Hettyey, A., Tóth, Z., Thonhauser, K. E., Frommen, J. G., Penn, D. J., & Van Buskirk, J. (2015). The relative importance of prey-borne and predator-borne chemical cues for inducible antipredator responses in tadpoles. *Oecologia*, *179*, 699–710. https://doi.org/10.1007/s00442-015-3382-7

Hettyey, A., Tóth, Z., & Van Buskirk, J. (2014). Inducible chemical defences in animals. *Oikos*, *123*, 1025–1028. https://doi.org/10.1111/oik.01338

Hettyey, A., Vincze, K., Zsarnóczai, S., Hoi, H., & Laurila, A. (2011). Costs and benefits of defences induced by predators differing in dangerousness. *Journal of Evolutionary Biology*, *24*, 1007–1019. https://doi.org/10.1111/j.1420-9101.2011.02233.x

Julious, S. A. (2004). Sample sizes for clinical trials with normal data. *Statistics in Medicine*, *23*, 1921–1986. https://doi.org/10.1002/sim.1783

Karban, R., & Baldwin, I. T. (1997). *Induced responses to herbivory*. Chicago, IL: Chicago University Press.

Kawecki, T. J., & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology Letters*, *7*, 1225–1241. https://doi.org/10.1111/j.1461-0248.2004.00684.x

Kicklighter, C. (2012). Chemical defences against predators. In C. Brönmark & L.-A. Hansson (Eds.), *Chemical ecology in aquatic systems* (pp. 236–249). Oxford, UK: Oxford University Press.

Kishida, O., & Nishimura, K. (2005). Multiple inducible defences against multiple predators in the anuran tadpole, *Rana pirica*. *Evolutionary Ecology Research*, *7*, 619–631.

Kishida, O., Trussell, G. C., & Nishimura, K. (2007). Geographic variation in a predator-induced defence and its genetic basis. *Ecology*, *88*, 1948–1954.

Kubanek, J., Whalen, K. E., Engel, S., Kelly, S. R., Henkel, T. P., Fenical, W., & Pawlik, J. R. (2002). Multiple defensive roles for triterpene glycosides from two Caribbean sponges. *Oecologia*, *131*, 125–136. https://doi.org/10.1007/s00442-001-0853-9

Kurali, A., Pásztor, K., Hettyey, A., & Tóth, Z. (2016). Toxin depletion has no effect on antipredator responses in common toad (*Bufo bufo*) tadpoles. *Biological Journal of the Linnean Society*, *119*, 1000–1010.

Lardner, B. (2000). Morphological and life history responses to predators in larvae of seven anurans. *Oikos*, *88*, 169–180. https://doi.org/10.1034/j.1600-0706.2000.880119.x

Laurila, A., Kujasalo, J., & Ranta, E. (1998). Predator-induced changes in life history in two anuran tadpoles: Effects of predator diet. *Oikos*, *83*, 307–317. https://doi.org/10.2307/3546842

Lenth, R. V. (2016). Least-square means: The R package lsmeans. *Journal of Statistical Software*, *69*, 1–33.

Magarlamov, T. Y., Melnikova, D. I., & Chernyshev, A. V. (2017). Tetrodotoxin producing bacteria: Detection, distribution and migration of the toxin in aquatic systems. *Toxins*, *5*, 166. https://doi.org/10.3390/toxins9050166

Magurran, A. E. (1990). The inheritance and development of minnow anti-predator behaviour. *Animal Behavior*, *39*, 834–842. https://doi.org/10.1016/S0003-3472(05)80947-9

Mangoni, M. L., Miele, R., Renda, T. G., Barra, D., & Simmaco, M. (2001). The synthesis of antimicrobial peptides in the skin of *Rana esculenta* is stimulated by microorganisms. *The FASEB Journal*, *15*, 1431–1432.

Manteifel, Y. B., & Reshetnikov, A. N. (2002). Avoidance of noxious tadpole prey by fish and invertebrate predators: Adaptivity of a chemical defence may depend on predator feeding habits. *Archiv Fur Hydrobiologie*, *153*, 657–668. https://doi.org/10.1127/archiv-hydrobiol/153/2002/657

McCoy, M. W., Touchon, J. C., Landberg, T., Warkentin, K. M., & Vonesh, J. R. (2012). Prey responses to predator chemical cues: Disentangling the importance of the number and biomass of prey consumed. *PLoS ONE*, *7*, e47495. https://doi.org/10.1371/journal.pone.0047495

Miele, R., Ponti, D., Boman, H. G., Barra, D., & Simmaco, M. (1998). Molecular cloning of a bombinin gene from *Bombina orientalis*: Detection of NF-UB and NF-IL6 binding sites in its promoter. *FEBS Letters*, *431*, 23–28.

Miner, B. G., Sultan, S. E., Morgan, S. G., Padilla, D. K., & Relyea, R. A. (2005). Ecological consequences of phenotypic plasticity. *Trends in Ecology & Evolution*, *20*, 685–692. https://doi.org/10.1016/j.tree.2005.08.002

Peacor, S. D. (2003). Phenotypic modifications to conspecific density arising from predation risk assessment. *Oikos*, *100*, 409–415. https://doi.org/10.1034/j.1600-0706.2003.12043.x

Pfennig, D. W., Wund, M. A., Snell-Rood, E. C., Cruickshank, T., Schlichting, C. D., & Moczek, A. P. (2010). Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology & Evolution*, *25*, 459–467. https://doi.org/10.1016/j.tree.2010.05.006

Relyea, R. A. (2001). The relationship between predation risk and antipredator responses in larval anurans. *Ecology*, *82*, 541–554. https://doi.org/10.1890/0012-9658(2001)082[0541:TRBPRA]2.0.CO;2

Ruxton, G. D., & Beauchamp, G. (2008). Time for some a priori thinking about post hoc testing. *Behavioral Ecology*, *19*, 690–693. https://doi.org/10.1093/beheco/arn020

Schoeppner, N. M., & Relyea, R. A. (2005). Damage, digestion, and defence: The roles of alarm cues and kairomones for inducing prey defences. *Ecology Letters*, *8*, 505–512. https://doi.org/10.1111/j.1461-0248.2005.00744.x

Semlitsch, R. D. (1993). Effects of different predators on the survival and development of tadpoles from the hybridogenetic *Rana esculenta* complex. *Oikos*, *67*, 40–46. https://doi.org/10.2307/3545093

Sih, A. (1986). Antipredator responses and the perception of danger by mosquito larvae. *Ecology*, *67*, 434–441. https://doi.org/10.2307/1938587

Slattery, M., Starmer, J., & Paul, V. J. (2001). Temporal and spatial variation in defensive metabolites of the tropical Pacific soft corals *Sinularia maxima* and *S. polydactyla*. *Marine Biology*, *138*, 1183–1193. https://doi.org/10.1007/s002270100540

Thornton, R. S., & Kerr, R. G. (2002). Induction of pseudopterosin biosynthesis in the gorgonian *Pseudopterogorgia elisabethae*. *Journal of Chemical Ecology*, *28*, 2083–2090.

Toledo, R. C., & Jared, C. (1995). Cutaneous poison glands and amphibian venoms. *Comparative Biochemistry and Physiology A*, *111*, 1–29.

Tollrian, R., Duggen, S., Weiss, L. C., Laforsch, C., & Kopp, M. (2015). Density-dependent adjustment of inducible defenses. *Scientific Reports*, *5*, 12736. https://doi.org/10.1038/srep12736

Tollrian, R., & Harvell, C. D. (1999). *The ecology and evolution of inducible defences*. Princeton, NJ: Princeton University Press.

Tóth, Z., Kurali, A., Móricz, Á. M., & Hettyey, A. (2019). Changes in toxin quantities following experimental manipulation of toxin reserves in *Bufo bufo* tadpoles. *Journal of Chemical Ecology*, *45*, 253–263. https://doi.org/10.1007/s10886-019-01045-9

Ujszegi, J., Móricz, Á., Krüzselyi, D., & Hettyey, A. (2017). Skin toxin production of toads changes during early ontogeny but is not adjusted to the microbiota of the aquatic environment. *Evolutionary Ecology*, *31*, 925–936. https://doi.org/10.1007/s10682-017-9920-5

Üveges, B., Hettyey, A., Móricz, Á. M., Drahos, L., Capon, R. J., Van Buskirk, J., … Bókony, V. (2019). Data: Predator-induced changes in the chemical defence of a vertebrate [Dataset]. figshare, https://doi.org/10.6084/m9.figshare.8256620.v1

Üveges, B., Fera, G., Móricz, Á. M., Krüzselyi, D., Bókony, V., & Hettyey, A. (2017). Age- and environment-dependent changes in chemical defences of larval and post-metamorphic toads. *BMC Evolutionary Biology*, *17*, 137. https://doi.org/10.1186/s12862-017-0956-5

Üveges, B., Szederkényi, M., Mahr, K., Móricz, Á. M., Krüzselyi, D., Bókony, V., … Hettyey, A. (2019). Chemical defence of toad tadpoles under risk by four predator species. *Ecology and Evolution*, *9*, 6287–6299.

Van Buskirk, J. (2000). The costs of an inducible defense in anuran larvae. *Ecology*, *81*, 2813–2821. https://doi.org/10.1890/0012-9658(2000)081[2813:TCOAID]2.0.CO;2

Van Buskirk, J. (2001). Specific induced responses to different predator species in anuran larvae. *Journal of Evolutionary Biology*, *14*, 482–489. https://doi.org/10.1046/j.1420-9101.2001.00282.x

Van Buskirk, J. (2014). Incipient habitat race formation in an amphibian. *Journal of Evolutionary Biology*, *27*, 585–592. https://doi.org/10.1111/jeb.12327

Van Buskirk, J., & Arioli, M. (2002). Dosage response of an induced defense: How sensitive are tadpoles to predation risk? *Ecology*, *83*, 1580–1585. https://doi.org/10.1890/0012-9658(2002)083[1580:DROAID]2.0.CO;2

Van Buskirk, J., Ferrari, M., Kueng, D., Näpflin, K., & Ritter, N. (2011). Prey risk assessment depends on conspecific density. *Oikos*, *120*, 1235–1239. https://doi.org/10.1111/j.1600-0706.2010.19311.x

West-Eberhard, M. J. (1989). Phenotypic plasticity and the origins of diversity. *Annual Review of Ecology and Systematics*, *20*, 249–278. https://doi.org/10.1146/annurev.es.20.110189.001341

West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*. Oxford, UK: Oxford University Press.

Yeaman, S., & Otto, S. (2011). Establishment and maintenance of adaptive genetic divergence under migration, selection, and drift. *Evolution*, *65*, 2123–2129. https://doi.org/10.1111/j.1558-5646.2011.01277.x

SUPPORTING INFORMATION

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