J Chem Ecol (2016) 42:329–338
DOI 10.1007/s10886-016-0690-2

Variation in Chemical Defense Among Natural Populations of Common Toad, *Bufo bufo*, Tadpoles: the Role of Environmental Factors

Veronika Bókony[1] · Ágnes M. Móricz[2] · Zsófia Tóth[3] · Zoltán Gál[1,4] · Anikó Kurali[1] · Zsanett Mikó[1] · Katalin Pásztor[1] · Márk Szederkényi[1,5] · Zoltán Tóth[1] · János Ujszegi[1] · Bálint Üveges[1,5] · Dániel Krüzselyi[2] · Robert J. Capon[6] · Herbert Hoi[5] · Attila Hettyey[1]

Received: 25 October 2015 / Revised: 20 January 2016 / Accepted: 29 March 2016 / Published online: 8 April 2016

Abstract Defensive toxins are widespread in nature, yet we know little about how various environmental factors shape the evolution of chemical defense, especially in vertebrates. In this study we investigated the natural variation in the amount and composition of bufadienolide toxins, and the relative importance of ecological factors in predicting that variation, in larvae of the common toad, *Bufo bufo*, an amphibian that produces toxins *de novo*. We found that tadpoles' toxin content varied markedly among populations, and the number of compounds per tadpole also differed between two geographical regions. The most consistent predictor of toxicity was the strength of competition, indicating that tadpoles produced more compounds and larger amounts of toxins when coexisting with more competitors. Additionally, tadpoles tended to contain larger concentrations of bufadienolides in ponds that were less prone to desiccation, suggesting that the costs of toxin production can only be afforded by tadpoles that do not need to drastically speed up their development. Interestingly, this trade-off was not alleviated by higher food abundance, as periphyton biomass had negligible effect on chemical defense. Even more surprisingly, we found no evidence that higher predation risk enhances chemical defenses, suggesting that low predictability of predation risk and high mortality cost of low toxicity might select for constitutive expression of chemical defense irrespective of the actual level of predation risk. Our findings highlight that the variation in chemical defense may be influenced by environmental heterogeneity in both the need for, and constraints on, toxicity as predicted by optimal defense theory.

Keywords Amphibian toxins · Bufadienolides · Geographical variation · Aquatic community · Pond permanence

Electronic supplementary material The online version of this article (doi:10.1007/s10886-016-0690-2) contains supplementary material, which is available to authorized users.

✉ Veronika Bókony
bokony.veronika@agrar.mta.hu

[1] Lendület Evolutionary Ecology Research Group, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman Ottó u. 15, H-1022 Budapest, Hungary

[2] Department of Pathophysiology, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman Ottó u. 15, H-1022 Budapest, Hungary

[3] Department of Evolutionary Zoology and Human Biology, University of Debrecen, Egyetem tér 1, H-4032 Debrecen, Hungary

[4] Agricultural Biotechnology Institute, NARIC, Szentgyörgyi Albert u. 4, H-2100 Gödöllő, Hungary

[5] Department of Integrative Biology and Evolution, Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Savoyenstrasse 1, 1160 Vienna, Austria

[6] Institute for Molecular Bioscience, University of Queensland, St Lucia, QLD 4072, Australia

Introduction

Noxious secondary metabolites are widespread in nature from unicellular organisms to plants and animals. These chemicals fulfill multiple ecological roles, acting as defenses against predators, competitors, parasites, and pathogens, or can be used for killing prey (Brodie 2009). Although the nature and functions of defensive toxins have been intensely studied in certain taxa such as plants, fungi, and marine invertebrates (Fritz and Simms 1992; McClintock and Baker 2001), we still know relatively little about how various environmental factors

shape the costs and benefits of chemical defense and ultimately its evolution, especially in vertebrates (Hettyey et al. 2014).

The optimal defense theory, originally developed for plants, predicts that the type and extent of chemical defense should depend on the trade-off between the need for defense and its costs (Fritz and Simms 1992; McCall and Fordyce 2010). On one hand, predictable environmental differences are expected to select for different levels of constitutive defenses, such that individuals (or body parts) exposed to more frequent interactions with enemies should be more toxic. On the other hand, less predictable spatial and temporal variation in the payoffs of toxicity is expected to favor phenotypic plasticity, such that organisms invest in defense only when/where its benefits outweigh its costs, resulting in inducible defenses. It is largely unexplored how this theory applies to the chemical defenses of vertebrates, a group that displays a complex and well known array of defenses in terms of behavior, morphology, and life history (Hettyey et al. 2014), but also with striking examples of toxicity (Arbuckle et al. 2013; Daly 1995; Ligabue-Braun and Carlini 2015).

Amphibian larvae offer opportunities to study the adaptive significance of chemical defenses for several reasons. First, many amphibians contain toxins that are present in early ontogenetic stages (Brossman et al. 2014; Daly 1995; Hayes et al. 2009a). Second, because amphibians often reproduce in a wide variety of water bodies, their offspring may find themselves in environments with very different types and quantities of enemies (Relyea 2002; Van Buskirk and Arioli 2005). This environmental heterogeneity may result in heterogeneous levels of constitutive toxicity, for example *via* differential availability of toxin precursors (*e.g.*, Darst et al. 2005) or *via* local genetic adaptations to the sensitivity of target organisms; *i.e.*, different chemicals may be needed against enemies with different toxin resistance (*e.g.*, Barlow et al. 2009; Crossland and Alford 1998). Third, since toxin production and storage requires specialized biochemical machinery, it is expected to be costly for developing larvae, especially when resources are limited. This combination of costliness with a stochastically varying need for investment creates favorable conditions for the evolution of inducible chemical defenses (Hettyey et al. 2014), especially if gene flow among populations hinders local genetic adaptation of toxicity (Sultan and Spencer 2002).

In this study, we aimed to explore the natural variation in chemical defense and its relationships with environmental factors in larvae of common toads, *Bufo bufo*. Bufonid toads synthesize cardiotonic steroids named bufadienolides that are highly toxic due to their membrane Na^+/K^+-ATPase antagonistic effect (Daly 1995). These toxins are found in low quantities in common toad hatchlings but are abundant in tadpoles (unpublished data), thus the larvae produce their toxins *de novo* with most, if not all, of their toxicity not being maternally inherited, in contrast to some other chemically defended amphibians (Hanifin et al. 2003; Hayes et al. 2009a). Reliance on chemical defense has long been suspected as the reason for common toad tadpoles usually showing little morphological or behavioral responses to predators (Richter-Boix et al. 2007; Van Buskirk 2002), thus raising the not yet tested possibility that they adjust their toxicity to predation risk. Additionally, the high philopatry of common toads (Reading et al. 1991) could facilitate local adaptations of constitutive chemical defenses, but whether such adaptations occur in nature also is untested.

We investigated the extent of variation in the amount and composition of bufadienolides among toad populations at both fine and coarse spatial scales, and the relative importance of ecological factors in predicting toxin variation. We focused on four factors that are likely to influence the payoff of chemical defense. On one hand, the two main benefits of toxicity can be protection from predators (Crossland and Alford 1998; Gunzburger and Travis 2005) and deterring, inhibiting or even killing competitors (Crossland and Alford 1998; Crossland and Shine 2012; Licht 1967), suggesting that higher levels of chemical defenses (production of larger quantities of toxins and/or greater number of toxin components) can be expected in populations in which predation risk is higher and/or competitors are more numerous. On the other hand, the costs of toxicity should depend on the constraints on resource allocation (*e.g.*, higher levels of chemical defense come at the cost of lower fat stores in diapausing butterflies; Fordyce et al. 2006), with such trade-offs being alleviated by abundant food. Therefore, chemical defense should be expected to increase with the amount of food available to tadpoles. Furthermore, allocating resources to chemical defense may be particularly costly for larvae that have to speed up their growth, for example, in cases in which the aquatic environment is prone to desiccation (Richter-Boix et al. 2011). In that case, reduced chemical defenses may be typical for tadpoles developing in less permanent aquatic habitats.

Methods

Data Collection During March–May 2014, we monitored 18 ponds in Hungary and 9 ponds in Austria where toads were likely to breed based on our observations in previous years. Out of these 27 ponds, we collected sufficient samples of toad tadpoles and environmental data from 19 ponds: 13 in Hungary and 6 in Austria (Supplementary 1). All ponds were located in wooded habitats, 200–550 m above sea level, with surface area and water depth ranging from 23–3253 m^2 and 6–90 cm, respectively (Supplementary 2).

Data collection at each pond was conducted when tadpoles had started to develop hind limbs, *i.e.*, stage 26–29 (mostly 27–28) according to Gosner (1960). We chose this developmental stage because we found that bufadienolide

concentration was highest in stage-26 toad tadpoles (unpublished data). To investigate chemical defenses, we randomly sampled 10 tadpoles per pond, and stored them individually in 1 ml 70 % HPLC-grade methanol until laboratory analysis; in 3 ponds, we collected only 8, 6, and 4 tadpoles, due to the low numbers present. To minimize the likelihood of sampling siblings, we collected tadpoles from several locations within each pond. We assessed developmental stage from an additional sample of 10 tadpoles per pond wherever possible, because tadpoles collected for toxin measurement had to be sacrificed immediately upon capture and their further examination was not possible without jeopardizing the validity of the chemical analysis. The percentages of tadpoles in stage 26, 27, 28, and 29 were 14.7, 57.3, 25.3, and 2.7 %, respectively. This slight variation in developmental stage was not associated with any of the variables analyzed below (*i.e.*, country, pond characteristics, and toxicity measures).

To estimate the density of tadpoles and their predators and competitors in each pond, we used both pipe sampling and dip-netting (Relyea 2002; Van Buskirk and Arioli 2005). For pipe sampling, we placed a 70-cm diam. and 100-cm long metal pipe vertically at randomly chosen points within each pond, and counted the animals captured within the pipe by repeatedly scooping with a net. We considered a pipe empty when five consecutive scoops did not capture any animals. We also collected samples by sweeping with dip nets having a triangular opening (37-cm long sides) at the bottom of the pond for a distance of 1 m, and counted all animals captured. Pipe sampling and dip netting covered a similar area of ca. 0.4 m^2. We took an equal, or close-to-equal, number of pipe samples and dip-net samples from each pond. Sample locations were chosen to reflect the diversity of microhabitats. The number of samples per pond increased with pond size and with the diversity of microhabitats (8–19 samples per pond).

We estimated food availability in the ponds by sampling periphyton, which is the main food source of tadpoles (Wells 2007). We placed 10-cm^2 glazed ceramic tiles on the bottom, 15–25 per pond depending on pond size, between 19 and 27 March, when toads were starting to breed. Approximately one and a half months later, when also sampling tadpoles, we collected the tiles and removed the periphyton by scraping with a razor blade and rinsing with distilled water. The resulting periphyton suspensions were stored at −20 °C until laboratory analysis.

To characterize the ponds' physical and chemical properties that are related to the probability of desiccation, we took various measurements when sampling the animals. We estimated: 1) the area of water surface as $A \times B \times \pi$, where A and B are the semi-major and semi-minor axes assuming an elliptical shape, 2) average water depth from 5 to 14 measurements (depending on pond size) taken at random points in the pond, 3) canopy cover as the % of pond surface that was overhung by trees, 4) submerged vegetation cover as the % of pond surface with visible water plants, 5) water temperature, dissolved oxygen, and conductivity as the averages from 3 to 5 randomly taken water samples (depending on pond size), measured by a portable electrochemistry meter (Consort C 6020 T; Consort Ltd., Turnhout, Belgium). Canopy cover and submerged vegetation cover were estimated by eye (*e.g.*, Van Buskirk and Arioli 2005) by the same three observers at each pond; we used the median of the 3 estimates for each pond.

Analysis of Bufadienolides We homogenized tadpoles using a VWR VDI 12 homogenizer with an IKA S12N-7S dispersing tool. We dried samples under vacuum at 45 °C using a rotary evaporator (Büchi Rotavapor R-134, Flawil, Switzerland), and measured dry mass to the nearest 0.0001 g using an Ohaus Pioneer PA-114 analytical balance. We redissolved the samples in 1 ml HPLC grade methanol, which was aided by brief use of ultrasound. Finally, we filtered samples using FilterBio nylon syringe filters (pore size = 0.22 μm).

We used high-performance liquid chromatography (LC) with diode-array detection and mass spectrometry (HPLC-DAD-MS) to identify and quantify bufadienolide compounds in tadpoles. We used a single quad LC-MS system (Model LC-MS-2020, Shimadzu, Kyoto, Japan) equipped with a binary gradient solvent pump, a vacuum degasser, a thermostated autosampler, a column oven, a photodiode detector, and a mass analyzer with electrospray ionization (ESI/MS). Chromatographic separation was carried out at 35 °C on a Kinetex C_{18} 2.6 μm column (100 × 3 mm i.d.) in series with an octadecyl C_{18} guard column (4 × 3 mm i.d.) using 10 μl injections. The mobile phase consisted of water containing 0.05 % HCOOH (solvent A) and acetonitrile containing 0.05 % HCOOH (solvent B). The flow rate was 0.8 $ml.min^{-1}$, and the gradient was as follows: 0–2 min., 15–25 % B; 2–15 min., 25–35 % B; 15–24 min., 30–50 % B; 24–25 min., 50–100 % B; 25–30 min., 100 % B; 30–35 min., 15 % B. ESI was operated under the following conditions: desolvation line (DL) temperature, 250 °C; heat block temperature, 400 °C; drying N_2 gas flow, 15 $l.min^{-1}$, nebulizer N_2 gas flow, 1.5 $l.min^{-1}$, positive ionization mode. Data were acquired and processed using the LabSolutions 5.42v program (Shimadzu, Kyoto, Japan).

We used commercially purchased bufalin, bufotalin, resibufogenin, gamabufotalin, arenobufagin, telocinobufagin (Biopurify Phytochemicals, Chengdu, China), cinobufagin (Chembest, Shanghai, China), cinobufotalin (Quality Phytochemicals, NJ, USA), and digitoxigenin (Santa Cruz Biotechnology, Dallas, TX, USA) as standards. Bufadienolides were recognized by their characteristic UV spectrum (Supplementary 3, Fig. S1). Identifications were carried out by comparing peak retention times and *m/z* values to those of standards and to the peaks present in two compound

mixes (Supplementary 3, Fig. S2). One compound mix was prepared by pooling tadpole samples from the 19 ponds (50 μl of each sample); the other compound mix was obtained by gently massaging the parotoid glands of 49 juvenile common toads originating from Silbersee, Vienna, Austria (48°12′33″ N, 16°15′48″E). We used compound mixes because they yielded much higher quantities of toxins and, therefore, higher-intensity absorption and mass spectra, than did individual tadpoles.

Quantitative evaluation was based on MS data. We used the calibration curve of the bufotalin standard to express the bufotalin-equivalent concentration of each bufadienolide compound per sample; these values then were divided by tadpole dry mass to obtain concentrations per tadpole mass (ng/mg; for similar approaches see Benard and Fordyce 2003; Hagman et al. 2009). Henceforth, we refer to this as total amount of budadienolides.

Measuring Periphyton Biomass The periphyton suspensions were dried at 105 °C for 20 hr, then diluted with 3 ml distilled water and homogenized with an ultrasonic shaker. Samples were dried again, until the dry weight stabilized, and weighed after cooling. Finally, samples were ashed at 550 °C for 2 hr, and weighed again after cooling. Ash-free dry mass was determined as the difference between dried and ashed sample mass; this represents all organic material that accumulated on the tile, including algae, bacteria, fungi, and particulate organic matter. We calculated periphyton biomass for each pond as the average ash-free dry mass of the tile samples (mg/tile).

Data Analyses All statistical analyses were run with R 3.1.0 (R Core Team 2014), using the packages 'nlme', 'AICcmodavg', and 'vegan'. All data are available in Supplementary 2.

For each pond, we calculated the average density of each sampled animal taxon as the average number of individuals across all pipe and dip-net samples divided by water volume (*i.e.*, the 0.38 m^2 bottom area multiplied by water depth). We quantified predation risk and the strength of competition as the weighted density of predators and competitors, respectively, in each pond. For predators, we used the estimates of relative danger derived from earlier studies (Crossland and Alford 1998; Henrikson 1990; Hettyey et al. 2011; Van Buskirk and Arioli 2005; Van Buskirk and Schmidt 2000) to weight the density of each taxon as follows: densities of larval aeshnid dragonflies (Aeshna sp. and Anax sp.), adults and larvae of *Dytiscus marginalis* waterbeetles, and adults of *Acilius* sp. waterbeetles were weighted by 3; densities of hemipteran (Notonecta sp.) adults, smooth newts (*Lissotriton vulgaris*), and alpine newts (*Ichthyosaura alpestris*) were weighted by 2; whereas the densities of larval libellulid dragonflies, hirudinid leeches, and small (<15 mm) water-dwelling spiders (*Dolomedes* sp.) were weighted by 1. For competitors, the densities of common toad, agile frog (*Rana dalmatina*) and common frog (*Rana temporaria*) larvae were weighted by 3 because they were of similar size and developmental stage; the density of European tree frog (*Hyla arborea*) tadpoles was weighted by 2 because they were considerably younger and smaller; and the densities of invertebrate taxa whose diet include periphyton were weighted by 1, specifically caddisfly larvae (Trichoptera), mayfly larvae (Ephemeroptera), isopods (*Asellus* sp.), and amphipods (Gammaridae). Thus, we calculated predation risk as: 3 × (aeshnids + Dytiscus + Acilius) + 2 × (Notonecta + Lissotriton + Ichthyosaura) + 1 × (Libellula + Hirudinidae + Dolomedes), and competitor density as: 3 × (*Bufo* + *Rana*) + 2 × Hyla + 1 × (Trichoptera + Ephemeroptera + Asellus + Gammaridae), whereby each taxon name stands for the density of that taxon per 1 m^3 water.

We summarized the variation in the seven measured physical and chemical pond characteristics using principal components analysis (PCA). The first axis explained 39 % of the variance and correlated positively with surface area, water depth, canopy cover, oxygen content, and conductivity, and correlated negatively with water temperature and % submerged vegetation (Supplementary 3, Table S1). Thus, ponds that were less prone to desiccation (*i.e.*, larger, deeper, cooler ponds with more canopy cover) received higher positive scores; henceforth, we refer to the scores along this PCA axis as "pond permanence".

We described the variation in chemical defenses with two variables: the number of bufadienolide compounds per tadpole and the total amount of bufadienolides per tadpole. We analyzed the effects of predation risk, competitor density, periphyton biomass, and pond permanence on chemical defenses by linear mixed-effects models (LME); the requirements of LME were checked by inspecting residual plots. The total amount of bufadienolides per tadpole, predation risk, competitor density, and periphyton biomass were $\log_{10}$-transformed to improve model fit. There was no multicolinearity as indicated by low values of variance inflation factor (VIF < 1.78), and no strong interaction among the 4 ecological variables (all two-way interactions $P > 0.256$).

The analysis of each dependent variable was done in two steps. First, we examined the random-effects structure of the data using the full models; *i.e.*, we included all 4 predictor variables as fixed effects, and compared the fit of models containing various random effects. Four random structures assumed that differences in chemical defense varied with the distance among ponds following a certain function (*i.e.*, Gaussian, exponential, spherical, or rational quadratic). Three random structures assumed that chemical defense varied among ponds, or between countries, or both, but these differences were not due to spatial distances. The null model assumed no random effects. We compared the fit of these 8 models by Akaike's information criterion (AIC), and for each

dependent variable we chose the model with the lowest AIC value.

In the second step, we used the random-effects structure selected in the first step, and quantified the relative importance of the fixed effects by information-theoretic model comparison based on AIC_c; *i.e.*, AIC corrected for sample size. We built a set of candidate models that contained various combinations of the four ecological predictors, and ranked the models by their AIC_c values. Since no model was clearly superior to the rest, we performed model-averaging, whereby we calculated the average estimate of each parameter across all models, weighted by the Akaike weight (ω) of the respective models. The 95 % confidence intervals, calculated from unconditional standard errors, of the model-averaged coefficients provided the non-standardized estimates of effect size for each predictor. As a standardized estimate of effect size that is comparable among the predictors within a model, we calculated the sum of Akaike weights (Σ_ω) across all models for each predictor, which expresses their relative importance.

Additionally, we also took an alternative approach to analyze the relationship between the composition of toad toxins and predators or competitors. For each pond, we calculated the % tadpoles that contained each bufadienolide and, based on these variables, we calculated dissimilarities between each pair of ponds using the Canberra distance, which is relatively less biased by the more abundant compounds than other dissimilarity metrics (Krebs 1999). We also constructed a dissimilarity matrix based on the non-weighted densities of each predator (8 taxa; the two newt species were counted as one because *I. alpestris* was not present in Hungarian ponds) or competitor (6 taxa; the two *Rana* species were pooled, and *H. arborea* was excluded because it was found only in a single pond). Then we used Mantel tests to estimate the correlation between the two matrices that describe, respectively, the ponds' clustering based on the frequencies of bufadienolides, and their clustering based on the composition of the predator or competitor community.

Results

In total, we found 16 bufadienolide compounds in the toad tadpoles (Table 1). The number of compounds per tadpole showed a strongly supported spatial structure, which was best accounted for by a difference between Hungary (mean ± SE: 13.10 ± 0.45) and Austria (10.62 ± 0.66), and variation among ponds irrespective of spatial distance (Table 2). Out of the 16 compounds, 7 were less abundant (*i.e.*, found in a smaller % of tadpoles) in Austria than in Hungary, with one compound missing entirely from the Austrian samples (Table 1). The total amount of bufadienolides per tadpole also showed a strongly supported spatial structure, which was best described by random variation among ponds irrespective of distance (Table 2). Although the spatial models (*i.e.*, assuming that differences in toxin concentration varied with the spatial distance between ponds) were also relatively well supported (Table 2), the difference between Hungary (31.68 ± 1.16 ng/mg) and Austria (34.96 ± 1.25 ng/mg) was not significant for the total amount of bufadienolides (Table 2).

AIC_c-based model comparisons showed that competitor density was an important predictor of the number of bufadienolide compounds per tadpole (Tables 3 and 4). First, the best-fitting model contained competitor density as the single predictor, and competitor density was included in all models with $AIC_c < 2$, which all had better fit than the null model (Table 3). Second, the relative importance of competitor density was 2–3 times higher than the importance of the other three predictors (Table 4). Third, the confidence interval for the model-averaged parameter estimate of competitor density did not include zero (Table 4), showing that the number of bufadienolides increased as competition got stronger, with ca. one (0.86) additional compound in response to a 10-fold increase in weighted competitor density (Fig. 1).

For the total amount of bufadienolides per tadpole, none of the four predictors received clear support (Tables 3 and 4). Although competitor density, pond permanence, and predation risk all appeared relatively more (ca. twice as) important than periphyton biomass, all their confidence intervals included zero (Table 4). Nevertheless, the best-fitting model included pond permanence and competitor density, and each of these two predictors were included in 3 out of 4 models with $AIC_c < 2$, which all had better fit than the null model (Table 3). Furthermore, the confidence intervals of both pond permanence and competitor density were very close to excluding zero (Table 4). Thus, tadpoles tended to contain larger amounts of bufadienolides in ponds with stronger competition and lower probability of desiccation (Fig. 2). In contrast, the effects of predation risk and periphyton biomass had high uncertainty (Table 4).

The clustering of the 19 ponds based on toxin composition (*i.e.*, % tadpoles containing each bufadienolide) was correlated with their clustering based on the composition of competitor community (*i.e.*, density of each taxon; Mantel test: $r = 0.39$, $P = 0.015$; Supplementary 3, Fig. S3). No correlation was found with the composition of predator community ($r = 0.04$, $P = 0.368$).

Discussion

We detected large variation in chemical defenses of toad larvae in natural ponds, with the number of different compounds in each tadpole ranging from 3 to 16, and the total amount of bufadienolides ranging from 1 to almost 200 ng/mg. This variation among similarly developed tadpoles showed a marked spatial structure, such that tadpoles within the same

Table 1 Bufadienolide compounds found in toad tadpoles, and the percentage of tadpoles in which each compound was detected

Compound	*m/z*	Retention time (min)	% occurrence	
			Austria	Hungary
Gamabufotalin	403	3.6	81	90
Telocinobufagin	403	8.9	100	99
Bufotalin	445	9.7	100	100
Bufalin*	387	14.7	36	74
Unidentified compound 1	417	4.5	90	92
Unidentified compound 2	417	5.1	100	100
Unidentified compound 3	415	6.1	100	98
Unidentified compound 4	403	6.7	93	97
Unidentified compound 5*	729	6.9	2	54
Unidentified compound 6	401	11.1	91	97
Unidentified compound 7*	757	17.2	0	34
Unidentified compound 8	573	18.7	100	97
Unidentified compound 9*	571	20.1	74	89
Unidentified compound 11*	367	21.7	57	93
Unidentified compound 12*	365	22.8	2	21
Unidentified compound 13*	601	24.5	36	76

Bufadienolides marked with an asterisk occurred less frequently in Austria than in Hungary (χ^2 tests with false discovery rate correction)

pond were more alike than tadpoles from different ponds, and tadpoles from the Hungarian ponds contained more compounds (but similar concentration in total) than tadpoles from the Austrian ponds. To our knowledge, this is the first documentation of geographical variation in chemical defense for amphibians that produce toxin compounds *de novo*, even though several accounts exist of among-population variation in species that sequester toxins from their diet (Daly 1995) or whose ability to synthesize toxins is ambiguous (Hanifin et al. 2003). Some part of the variation among ponds is likely to reflect differences among families, as different families breed at different ponds. Nevertheless, the results showed that a considerable part of bufadienolide variation was attributable to differences among ponds in certain ecological characteristics, suggesting that toads with different toxicity are non-randomly distributed among ponds. Although random neutral processes, such as genetic drift, may contribute to this pattern, the correlative findings also could result from local adaptations and/or phenotypic plasticity. Below we speculate on these aspects of the results to provide a basis for future experimental work.

The most consistent predictor of toxicity was the strength of competition, which increased both the number of compounds and, to a lesser extent, the total amount of bufadienolides. These relationships were corroborated by the results of the cluster analysis, showing that ponds with similar toxin composition had similar competitor communities. These findings supported the hypothesis that toad tadpoles may alter toxin production in response to competition (Hettyey et al. 2014), producing larger amounts of toxins when competition

Table 2 Akaike's information criterion values of linear mixed-effects models with various random effects structures

Random effects	Number of compounds	Total amount of bufadienolides
None	799.2 [a]	87.0 [a]
Country	757.2 [b]	89.0 [a]
Pond	709.8 [c]	**46.3** [b]
Pond nested in country	**707.2** [d]	48.3 [b]
Gaussian spatial correlation	759.4 [b]	47.4 [b]
Exponential spatial correlation	711.4 [c]	47.8 [b]
Spherical spatial correlation	711.0 [c]	47.5 [b]
Rational quadratic spatial correlation	759.4 [b]	47.8 [b]

Each model included predation risk, competitor density, periphyton biomass, and pond permanence as fixed effects. Upper-case letters indicate the results of likelihood ratio tests: model fit differs between models marked with different letters. The best-fitting models are highlighted in bold

Table 3 Linear mixed-effects models ranked by Akaike's information criterion corrected for sample size (AIC_c), the number of estimated parameters (K), the AIC_c difference from the model with the lowest AIC_c (ΔAIC_c), and the Akaike weight (ω) shown for each model, with predation risk (PRED), competitor density (COMP), periphyton biomass (FOOD), and pond permanence (POND) as predictor variables

Predictors	K	AIC_c	ΔAIC_c	ω
a) Number of bufadienolide compounds				
COMP	5	708.73	0.00	0.22
POND + COMP	6	709.04	0.31	0.19
FOOD + COMP	6	710.44	1.70	0.09
POND + FOOD + COMP	7	710.46	1.73	0.09
COMP + PRED	6	710.53	1.80	0.09
NULL	4	711.12	2.39	0.07
POND + COMP + PRED	7	711.20	2.47	0.06
FOOD + COMP + PRED	7	712.31	3.57	0.04
POND	5	712.44	3.71	0.03
POND + FOOD + COMP + PRED	8	712.65	3.92	0.03
FOOD	5	713.15	4.42	0.02
PRED	5	713.21	4.48	0.02
POND + PRED	6	714.22	5.48	0.01
POND + FOOD	6	714.44	5.71	0.01
FOOD + PRED	6	715.26	6.53	0.01
POND + FOOD + PRED	7	716.14	7.40	0.01
b) Total amount of bufadienolides				
POND + COMP	5	26.29	0.00	0.19
POND	4	26.78	0.49	0.15
POND + COMP + PRED	6	27.18	0.89	0.12
COMP + PRED	5	27.79	1.50	0.09
POND + FOOD + COMP	6	28.43	2.14	0.06
PRED	4	28.58	2.29	0.06
NULL	3	28.65	2.36	0.06
POND + PRED	5	28.67	2.38	0.06
POND + FOOD	5	28.81	2.52	0.05
POND + FOOD + COMP + PRED	7	29.32	3.03	0.04
FOOD + COMP + PRED	6	29.88	3.59	0.03
COMP	4	30.04	3.75	0.03
FOOD + PRED	5	30.50	4.21	0.02
POND + FOOD + PRED	6	30.63	4.34	0.02
FOOD	4	30.75	4.45	0.02
FOOD + COMP	5	32.13	5.84	0.01

Models for each dependent variable included the respective random effects shown in bold in Table 2

is stronger and/or a greater variety of compounds when the community of competitors is more diverse. *Bufo* and *Rana* tadpoles were by far the most numerous group among the competitors found in the ponds (2.3–101,300 tadpoles/m^3, median = 1367, *vs.* 0–1321 invertebrate competitors/m^3, median = 0.7), suggesting that much of the relationship between toxicity and competitor density may be due to tadpole density.

Table 4 Relative importance (Σ_ω) of four ecological predictors, and their model-averaged parameter estimates (b_{av}) with 95 % confidence intervals (CI)

Model set	Σ_ω	b_{av}	95 % CI
a) Number of bufadienolide compounds			
Predation risk*	0.27	−0.14	−1.66, 1.38
Competitor density*	0.81	0.86	0.16, 1.56
Periphyton biomass*	0.30	−0.87	−3.37, 1.63
Pond permanence	0.43	0.32	−0.12, 0.76
b) Total amount of bufadienolides*			
Predation risk*	0.44	−0.13 (0.74)	−0.34, 0.08 (0.46, 1.20)
Competitor density*	0.57	0.08 (1.20)	−0.02, 0.18 (0.95, 1.51)
Periphyton biomass*	0.25	0.03 (1.07)	−0.31, 0.37 (0.49, 2.34)
Pond permanence	0.69	0.06 (1.15)	0, 0.12 (1.00, 1.32)

Variables marked with asterisks were $\log_{10}$-transformed before analysis; parameter estimates refer to the transformed values. For the total amount of bufadienolides, values in parentheses are back-transformed and show the proportional change in the original values (ng/mg)

Chemical interference among amphibian larvae has long been suggested to occur, but the nature of the involved agents (*e.g.*, metabolic waste products, specific growth inhibitors, or facultatively parasitic algae as mediators) is still subject to controversy (Licht 1967; Wells 2007). Toxins have rarely been considered as agents of interference competition (but see Crossland 1998; Crossland and Shine 2012), so it remains to be tested experimentally whether tadpoles increase toxin production as a phenotypically plastic response to crowding, and how such a response affects their competitors.

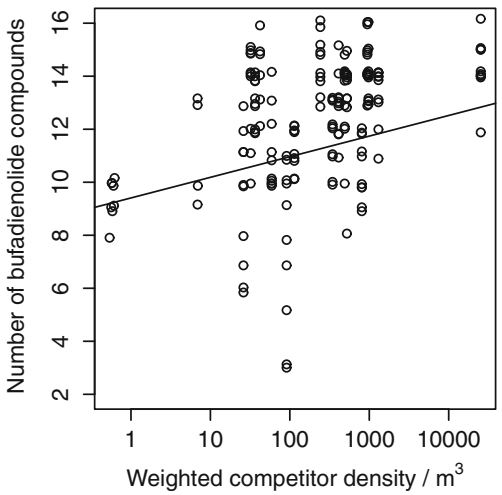

Fig. 1 Relationship between the number of bufadienolide compounds per tadpole and the weighted density of competitors. Overlapping data points were jittered; the regression line was fitted from a linear mixed-effects model with pond nested within country as random effects

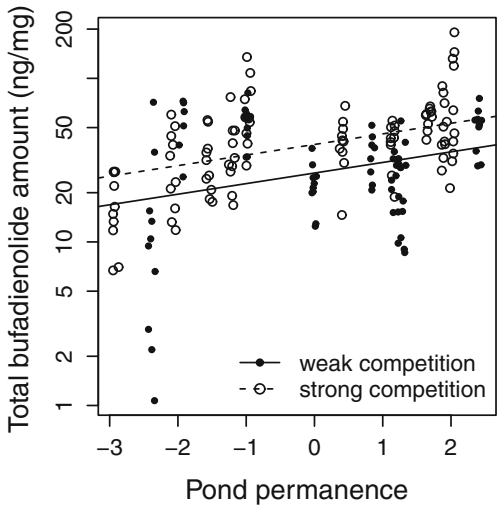

Fig. 2 Relationship between the total amount of bufadienolides per tadpole and pond permanence (Principal Component Analysis scores, with higher values indicating lower probability of desiccation). For illustrative purposes, ponds were divided into two groups according to whether the weighted density of competitors was lower (*i.e.*, weak competition) or at least as high (*i.e.*, strong competition) as the median ($242/m^3$); regression lines were fitted for these two groups from a LME model with pond as random effect. Overlapping data points were jittered

An alternative explanation for the positive correlation between toxin diversity and competitor density could be that more toxic tadpoles have higher survival probabilities (because of greater anti-predatory protection), leading to increased competitor densities in ponds where toads had higher toxicity due to random variation or local adaptation to predators. In this case, the competition effect would be attributable solely to the density of the toad tadpoles. This was not supported by our data (Supplementary 3, Table S2); compared to the effect of total competitor density on the number of compounds, *Bufo* density had less than half the effect, with zero well within its confidence interval. Thus, it is unlikely that higher densities were the result of higher toad toxicity (furthermore, see below on the effect of predators).

The other ecological predictor that emerged in the study as relatively important was pond permanence: tadpoles tended to contain larger concentrations of bufadienolides in ponds that were less likely to dry out. This relationship supports the hypothesis that toxin production has costs that can be afforded only by tadpoles that develop in relaxed conditions. The risk of desiccation, such as decreasing water levels, has been shown to speed up larval development in several amphibian species, but coming at a cost of reduced size (Richter-Boix et al. 2011). This was a risk for the tadpoles in our study, as one of the ponds we had monitored dried out completely after toad spawning, and some shrank to a few percent of their early-spring size. Thus, tadpoles that have to finish development and metamorphosis within a shorter time may have less to allocate into other energetically costly processes, such as synthesis of toxin compounds. Interestingly, this apparent trade-off was not alleviated by higher food abundance, as suggested by our result that periphyton biomass had negligible effect on chemical defense, despite the importance of this food source for tadpole development. However, because we monitored ponds that were known to be used consistently by toads as breeding sites and that harbor relatively large tadpole populations, our samples may have been biased toward high quality habitats in terms of tadpole food. It thus is possible that the variation within these habitats (0.8–9.5 mg periphyton/tile) was not limiting enough to constrain bufadienolide synthesis. Experimental studies are needed to ascertain whether food availability affects amphibian chemical defenses.

Surprisingly, our results did not support the hypothesis that predation risk enhances chemical defenses, as weighted predator density had a highly uncertain and, if anything, negative effect. This might be due to the fact that most of the predators present in our study ponds were invertebrates, which may be less sensitive to toad toxins than vertebrates. For example, a meta-analysis found that fish and salamanders were twice as likely to find amphibian prey (larvae or eggs) unpalatable than insect predators, and prey that commonly coexist with fish were more likely to be found unpalatable by fish predators (Gunzburger and Travis 2005). However, we found no relationship between the measures of chemical defense and the density of newts, the only vertebrate predators present in our ponds (Supplementary 3, Fig. S4). As an alternative explanation for the lack of predator effects, it is possible that chemical defense shows little plasticity in response to variation in predation risk if this variation is poorly predictable (*e.g.*, some predators, such as adult insects and newts, can move between ponds) and the cost of error is high (*i.e.*, undefended tadpoles are eaten). Because the synthesis of bufadienolides takes time, tadpoles may, instead, have to rely on prompt behavioral changes when responding to frequently varying predation risk (Marquis et al. 2004). In contrast, the community of competitors may be relatively predictable during tadpole ontogeny (*i.e.*, competitors do not move between ponds), and not responding to them may not be immediately life threatening. Therefore, we might expect more plasticity in toxin production in response to competitors, and a rather constitutive level of chemical defense against predators. The idea of constitutive anti-predatory toxicity is in line with recent experimental studies that exposed amphibian larvae to predatory cues and found either no effect on larval chemical defense (Benard and Fordyce 2003; Brossman et al. 2014) or contradicting effects on the chemical defense of froglets after metamorphosis (Benard and Fordyce 2003; Hagman et al. 2009). However, in these studies, larval toxins were absent or non-detectable (Benard and Fordyce 2003) or probably maternally inherited (Brossman et al. 2014), so further work is needed, particularly on taxa that are known to produce toxins, to assess the importance of predator-induced plasticity and locally adapted constitutive defenses.

Finally, it is notable that there was large individual variation even around the strongest relationships we found, leaving ample room for other potential predictors of chemical defense that were not investigated here. For example, certain toxic compounds of toads are known to have antimicrobial effects (Cunha Filho et al. 2005; Tempone et al. 2008); thus, variation among ponds in pathogen community may also influence the chemical defenses of tadpoles. Given the recent spread of amphibian diseases, the role of defensive chemicals as part of the innate immune response definitely deserves more attention (Woodhams et al. 2006). Interestingly, our results are mirrored by experimental findings on antimicrobial peptides, showing a strong positive effect of competition and a minor negative effect of predation risk on the level of defense (Groner et al. 2014). Furthermore, the marked spatial structure we found suggests that the genetic background and/or maternal effects may contribute to the variation in toxin production, perhaps as a result of selection for constitutive defenses according to the spatial variation in the type, intensity, and predictability of predation risk in various life-history stages. A further intriguing possibility is that *in situ* biotransformation by the bacterial flora living in amphibians may contribute to toxin diversity (Hayes et al. 2009b). Studies delving into these details will reveal much about how evolution fine-tunes the constitutive and inducible chemical defenses of animals with complex life histories.

Acknowledgments We thank Kutyó L. Jókai and Gábor Fera for help in the field, the Pilisi Parkerdő Zrt. for allowing us to use their forestry roads, and Edina Simon for help with the lab measurements of periphyton biomass. The Közép-Duna-Völgyi KTVF issued the permission to conduct the present study (KTF:603–3/2014). Financial support was provided by the 'Lendület' programme of the Hungarian Academy of Sciences (MTA, LP2012-24/2012), an FP7 Marie Curie Career Integration Grant (PCIG13-GA-2013–631722), and a Sparkling Science Project of the Federal Ministry of Science and Research, Austria (BMWF, SPA 04/171). During the study, Z. T. was supported by the MTA postdoctoral research programme (SZ-029/2013) and the Hungarian Scientific Research Fund (OTKA, PD108938). During write-up, V.B. was supported by the Bolyai Fellowship of the Hungarian Academy of Sciences.

References

Arbuckle K, Brockhurst M, Speed MP (2013) Does chemical defence increase niche space? A phylogenetic comparative analysis of the Musteloidea. Evol Ecol 27:863–881

Barlow A, Pook CE, Harrison RA, Wüster W (2009) Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. Proc R Soc B 276:2443–2449

Benard MF, Fordyce JA (2003) Are induced defenses costly? Consequences of predator-induced defenses in western toads, *Bufo boreas*. Ecology 84:68–78

Brodie ED (2009) Toxins and venoms. Curr Biol 19:R931–R935

Brossman KH, Carlson BE, Stokes AN, Langkilde T (2014) Eastern newt (*Notophthalmus viridescens*) larvae alter morphological but not chemical defenses in response to predator cues. Can J Zool 92: 279–283

Crossland MR, Alford RA (1998) Evaluation of the toxicity of eggs, hatchlings and tadpoles of the introduced toad *Bufo marinus* (Anura: Bufonidae) to native Australian aquatic predators. Aust J Ecol 23:129–137

Crossland MR, Shine R (2012) Embryonic exposure to conspecific chemicals suppresses cane toad growth and survival. Biol Lett 8: 226–229

Cunha Filhoa GA, Schwartz CA, Resck IS, Murta MM, Lemos SS, Castro MS, Kyaw C, Pires OR Jr, Leite JRS (2005) Antimicrobial activity of the bufadienolides marinobufagin and telocinobufagin isolated as major components from skin secretion of the toad *Bufo rubescens*. Toxicon 45:777–782

Daly JW (1995) The chemistry of poisons in amphibian skin. Proc Natl Acad Sci U S A 92:9–13

Darst CR, Menéndez-Guerrero PA, Coloma LA, Cannatella DC (2005) Evolution of dietary specialization and chemical defense in poison frogs (Dendrobatidae): a comparative analysis. Am Nat 165:56–69

Fordyce JA, Nice CC, Shapiro AM (2006) A novel trade-off of insect diapause affecting a sequestered chemical defense. Oecologia 149: 101–106

Fritz RS, Simms EL (1992) Plant resistance to herbivores and pathogens: ecology, evolution, and genetics. University of Chicago Press, Chicago

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190

Groner ML, Rollins-Smith LA, Reinert LK, Hempel J, Bier ME, Relyea RA (2014) Interactive effects of competition and predator cues on immune responses of leopard frogs at metamorphosis. J Exp Biol 217:351–358

Gunzburger MS, Travis J (2005) Critical literature review of the evidence for unpalatability of amphibian eggs and larvae. J Herpetol 39:547–57

Hagman M, Hayes RA, Capon RJ, Shine R (2009) Alarm cues experienced by cane toad tadpoles affect post-metamorphic morphology and chemical defences. Funct Ecol 23:126–132

Hanifin CT, Brodie ED III, Brodie ED Jr (2003) Tetrodotoxin levels in eggs of the rough-skin newt, *Taricha granulosa*, are correlated with female toxicity. J Chem Ecol 29:1729–1739

Hayes RA, Crossland MR, Hagman M, Capon RJ, Shine R (2009a) Ontogenetic variation in the chemical defences of cane toads (*Bufo marinus*): toxin profiles and effects on predators. J Chem Ecol 35: 391–399

Hayes RA, Piggott AM, Dalle K, Capon RJ (2009b) Microbial biotransformation as a source of chemical diversity in cane toad steroid toxins. Bioorg Med Chem Lett 19:1790–1792

Henrikson B-I (1990) Predation on amphibian eggs and tadpoles by common predators in acidified lakes. Holarct Ecol 13:201–206

Hettyey A, Vincze K, Zsarnóczai S, Hoi H, Laurila A (2011) Costs and benefits of defences induced by predators differing in dangerousness. J Evol Biol 24:1007–1019

Hettyey A, Tóth Z, Van Buskirk J (2014) Inducible chemical defences in animals. Oikos 123:1025–1028

Krebs CJ (1999) Ecological methodology, 2nd ed. Addison-Wesley Educational Publishers, Inc

Licht LE (1967) Growth inhibition in crowded tadpoles: intraspecific and interspecific effects. Ecology 48:736–745

Ligabue-Braun R, Carlini CR (2015) Poisonous birds: a timely review. Toxicon 99:102–108

Marquis O, Saglio P, Neveu A (2004) Effects of predators and conspecific chemical cues on the swimming activity of *Rana temporaria* and *Bufo bufo* tadpoles. Arch Hydrobiol 160:153–170

McCall AC, Fordyce JA (2010) Can optimal defence theory be used to predict the distribution of plant chemical defences? J Ecol 98:985–992

McClintock JB, Baker BJ (2001) Marine chemical ecology. CRC Press, Boca Raton

R Core Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Reading CJ, Loman J, Madsen T (1991) Breeding pond fidelity in the common toad, *Bufo bufo*. J Zool (Lond) 225:201–211

Relyea RA (2002) Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. Ecol Monogr 72: 77–93

Richter-Boix A, Llorente GA, Montori A (2007) A comparative study of predator-induced phenotype in tadpoles across a pond permanency gradient. Hydrobiologia 583:43–56

Richter-Boix A, Tejedo M, Rezende EL (2011) Evolution and plasticity of anuran larval development in response to desiccation. A comparative analysis. Ecol Evol 1:15–25

Sultan SE, Spencer HG (2002) Metapopulation structure favors plasticity over local adaptation. Am Nat 160:271–283

Tempone AG, Carvalho PD, Lebrun I, Sartorelli P, Taniwaki NN, de Andrade HF Jr, Antoniazzi MM, Jared C (2008) Antileishmanial and antitrypanosomal activity of bufadienolides isolated from the toad *Rhinella jimi* parotoid macrogland secretion. Toxicon 52:13–21

Van Buskirk J (2002) A comparative test of the adaptive plasticity hypothesis: relationships between habitat and phenotype in anuran larvae. Am Nat 160:87–102

Van Buskirk J, Arioli M (2005) Habitat specialization and adaptive phenotypic divergence of anuran populations. J Evol Biol 18:596–608

Van Buskirk J, Schmidt BR (2000) Predator-induced phenotypic plasticity in larval newts: trade-offs, selection, and variation in nature. Ecology 81:3009–3028

Wells KD (2007) The ecology and behavior of amphibians. University of Chicago Press, Chicago

Woodhams DC, Rollins-Smith LA, Carey C, Reinert L, Tyler MJ, Alford RA (2006) Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. Oecologia 146:531–540