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Cite this article: Bókony V, Mikó Z, Móricz ÁM., Krüzselyi D, Hettyey A. 2017 Chronic exposure to a glyphosate-based herbicide makes toad larvae more toxic. *Proc. R. Soc. B* **284**: 20170493.
http://dx.doi.org/10.1098/rspb.2017.0493

Received: 8 March 2017
Accepted: 1 June 2017

Subject Category:
Global change and conservation

Subject Areas:
environmental science, ecology

Keywords:
bufadienolides, chemical defence, ecotoxicology, pesticides, phenotypic plasticity

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Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3810586.

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Chronic exposure to a glyphosate-based herbicide makes toad larvae more toxic

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Chemical pollutants can exert various sublethal effects on wildlife, leading to complex fitness consequences. Many animals use defensive chemicals as protection from predators and diseases, yet the effects of chemical contaminants on this important fitness component are poorly known. Understanding such effects is especially relevant for amphibians, the globally most threatened group of vertebrates, because they are particularly vulnerable to chemical pollution. We conducted two experiments to investigate how exposure to glyphosate-based herbicides, the most widespread agrochemicals worldwide, affects the production of bufadienolides, the main compounds of chemical defence in common toads (*Bufo bufo*). In both experiments, herbicide exposure increased the amount of bufadienolides in toad tadpoles. In the laboratory, individuals exposed to 4 mg a.e./L glyphosate throughout their larval development had higher bufadienolide content at metamorphosis than non-exposed tadpoles, whereas exposure for 9 days to the same concentration or to 2 mg a.e./L throughout larval development or for 9 days had no detectable effect. In outdoor mesocosms, tadpoles from 16 populations exhibited elevated bufadienolide content after three-weeks exposure to both concentrations of the herbicide. These results show that pesticide exposure can have unexpected effects on non-target organisms, with potential consequences for the conservation management of toxin-producing species and their predators.

1. Introduction

We live in an era of environmental pollution, with a broad array of chemical contaminants, such as pesticides, heavy metals, and road de-icers being introduced into the environment in ever-growing quantities. Besides causing mortality events, these contaminants can also exert a variety of sublethal effects, sometimes even at very low concentrations, including the disruption of physiological functions such as endocrine, chemosensory, and immune systems, and the impairment of various behaviours related to feeding, predator avoidance, and reproduction [1,2]. Such effects can have far-reaching consequences by accumulating over time and across trophic levels, interacting with other stressors, and altering biotic relationships in natural communities [1–4].

One important component of fitness which may be affected by pollutants is chemical defence. Many groups of animals produce toxic compounds or sequester noxious metabolites from their diet for protection from predators, competitors, parasites, and pathogens [5,6]. These chemical defences may be disrupted by chemical contaminants, although the effect is sometimes, counter-intuitively, positive [3]. Among vertebrates, chemical defence is the most widespread in amphibians, a group of serious conservation concern due to their ongoing population declines worldwide [7]. In recent years, amphibian chemical defences have attracted increasing attention due to their potential to provide critical protection from emerging infectious diseases that are suspected to be one of the main drivers of global biodiversity loss [7]. Particular focus has been directed on the defensive role of antimicrobial skin peptides against chytridiomycosis, a spreading lethal disease caused by the fungus *Batrachochytrium dendrobatidis*, and the effects of

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contaminants on this defence [8–12]. These studies, however, yielded controversial results, reporting both positive and negative effects by various pollutants, as well as no effects on the production and bioactivity of skin peptides or on the animals' resistance to chytrid infection [8–12].

Bufadienolides are steroid compounds that inhibit Na^+/K^+-ATPases [13]. They are present in mammalian tissues functioning in blood pressure regulation and cell signalling [14], and due to their cardiotonic effect, they are used as chemical defence by various plants, fireflies, toads, and toad-eating snakes [13]. Similarly to skin peptides, bufadienolides have antimicrobial effects [15,16], but they also make their hosts distasteful or poisonous to predators [17], which is considered to be their main function. Toads rely on their bufadienolide defences from early on during their ontogeny [18,19] and show very little anti-predatory defences in terms of morphology and behaviour that are typical for other amphibian larvae [20,21]. Furthermore, predators with no shared evolutionary history with toads can be very sensitive to bufadienolides, as demonstrated by the dramatic lethal poisoning effects of the invasive cane toad (*Rhinella marina*) on native Australian wildlife [22]. Thus, understanding how environmental pollutants affect chemical defences can be crucially relevant for the protection or management of toxic species as well as their predators. However, to the best of our knowledge, no study has ever tested the effect of any chemical contaminant on the production of bufadienolides as a form of chemical defence.

In this study, we investigated how the bufadienolide content of common toad (*Bufo bufo*) larvae is affected by a glyphosate-based herbicide (GBH) formulation. GBHs are currently the largest selling agrochemicals in the world and ubiquitously contaminate natural water bodies [23–25]. They typically consist of the active ingredient glyphosate and surfactant additives, and have been shown to exert both lethal and sublethal effects in many species [26–28]. In tadpoles of the common toad, we found that a GBH formulation reduced survival and growth, and slowed down development, with younger individuals being more sensitive [29]. Because the results of ecotoxicological studies may strongly depend on the experimental venue [30], we combined two approaches to test the effects of GBH exposure on the toad tadpoles' bufadienolide chemical defence. First, we conducted an experiment under controlled laboratory conditions, contrasting the effects of short versus long exposure and tested whether the effect of short exposure depends on its ontogenetic timing. Second, we performed an experiment in a more natural outdoor mesocosm set-up, in which we investigated the effect of chronic GBH exposure in tadpoles originating from several different populations, because both chemical defence [19] and susceptibility to pesticides [31,32] can vary across populations.

2. Material and methods

We used a popular GBH formulation, Glyphogan® Classic (Monsanto Europe S.A., Brussels, Belgium) which contains 41.5 w/w% glyphosate and 15.5 w/w% polyethoxylated tallowamine surfactant. In both experiments, we applied the herbicide at three nominal concentrations, corresponding to 0, 2, and 4 mg a.e./L glyphosate. We chose these concentrations based on two earlier experiments that consistently showed that the LC_{50} value over 5 days of exposure was 4.4 mg a.e./L for toad tadpoles [33]. We did not measure the actual concentrations in the experimental containers in this study, but in our earlier work using the same protocols we measured 1.41 ± 0.34 (s.e.) and 1.57 ± 0.29 mg a.e./L in the laboratory and in the mesocosms, respectively, treated with the nominal concentration of 2 mg a.e./L [30]. These values are similar to the expected environmental concentration after application of certain GBHs at the maximum allowed label rate [24], whereas glyphosate concentrations up to 5.2 mg l^{-1} were found in run-off after GBH use [23]. The concentrations given throughout the text henceforward are nominal.

(a) Laboratory experiment

On 28 March 2014, we collected 70 eggs from each of 12 freshly laid clutches from a pond in Nagykovácsi, Hungary (47°34′35″ N, 18°52′06″ E), and transported them to the Evolutionary Ecology Laboratory at the Experimental Station of MTA ATK NÖVI in Julianna-major, Budapest (47°32′52″ N, 18°56′05″ E), where we maintained a 12 L : 12 D cycle throughout the experiment. Until hatching, we kept the eggs at 20°C separated by family in 3 l containers holding 1 l reconstituted soft water (RSW; 48 mg $NaHCO_3$, 30 mg $CaSO_4 \times 2\ H_2O$, 61 mg $MgSO_4 \times 7\ H_2O$, 2 mg KCl added to 1 l soft water). We started the experiment when the hatchlings reached the free-swimming state, i.e. developmental stage 25 [34] by haphazardly selecting 52 healthy-looking larvae from each clutch and placing them into the experimental containers. We reared the tadpoles individually at 18°C in 1 l containers filled with 0.7 l RSW, arranged in a randomized block design. We changed the rearing water every third day, and fed the tadpoles ad libitum with chopped and slightly boiled spinach (commercially bought frozen spinach for human consumption, hence unlikely to be contaminated with considerable amounts of pesticides or other toxicants).

We distributed the 624 tadpoles evenly across 13 treatment groups, such that we had four replicates in each treatment by family combination (i.e. four individually housed tadpoles × 13 treatments × 12 families). In the control treatment, we kept the tadpoles in GBH-free RSW throughout the experiment. The other 12 treatment groups form a 2 × 6 design, in which we combined the 2 GBH concentrations (i.e. low and high) with 6 different exposure times. The tadpoles were exposed to the GBH either during the entire duration of the experiment (until the start of metamorphosis; 36–61 days, mean: 44.27 ± 0.21 s.e.) or only for a 9-day period during the 1st, 2nd, 3rd, 4th, or 5th period of their larval development (i.e. days 1–9, 10–18, 19–27, 28–36, and 37–45, respectively). In the 10 treatment groups that were exposed to GBH for 9 days, we reared the tadpoles in GBH-free RSW outside the period of GBH exposure. During GBH exposure, we renewed the initial pesticide concentration (i.e. 1.11 or 2.22 ml of the herbicide, respectively, was added to 200 l RSW) at each water change.

To quantify toxin levels in a way that is comparable across all treatment groups, we measured bufadienolides after the end of the 5th 9-day period, at the onset of metamorphosis (developmental stage 42, [34]). We randomly selected five individuals from each treatment group (one from each of five families; $N = 65$ in total, i.e. 1 tadpole × 13 treatments × 5 families) and stored each in 1 ml 70% HPLC-grade methanol for chemical analysis. The rest of the tadpoles were kept alive as part of another experiment [29].

(b) Mesocosm experiment

Between 7 and 13 April 2015, we collected 40 eggs from each of eight freshly laid clutches from each of the 16 sites around Budapest, Hungary (electronic supplementary material, table S1), and transported them to the Julianna-major Experimental Station. Until hatching, we kept the eggs in the laboratory separated

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by family in 3 l containers holding 1 l of RSW at 20°C and a 12 L : 12 D cycle.

Two weeks before the start of the experiment, we placed 90 l plastic tubs in an open outdoor area and filled each of them with 65 l tap water. Two days later, we added 1 l pond water and 40 g dried beech (*Fagus sylvatica*) leaves to each tub to set up a self-sustaining ecosystem that provided nutrients and refuges for tadpoles [30,35]. To prevent colonization by predators, we covered the tubs with mosquito net lids. One day before the start of the experiment, we added 0.361 or 0.723 ml of the herbicide into the tubs belonging to the low or high GBH treatment group, respectively; the GBH concentrations were not renewed during the mesocosm experiment.

We started the experiment 2 days after the hatchlings reached developmental stage 25, by placing 24 haphazardly selected healthy-looking individuals into each tub. All animals in a tub originated from the same population, and we had four replicates for each population in each GBH treatment group (i.e. 4 tubs × 3 GBH concentrations × 16 populations); the treatments were assigned to the 192 tubs in a randomized block design. We measured bufadienolides 18 days after the start of the experiment, when the tadpoles were in developmental stages 32–35, most of them in stage 34 (mean: 33.76 ± 0.85 s.d.). We chose this stage to maximize the detectability of treatment effects, because in our earlier experiment we found that developing toads had the highest amount of bufadienolides around stage 34, and rearing conditions had the largest effect on toxin levels during this stage [36]. From each tub, we collected two randomly selected tadpoles, and stored them individually in 1 ml 70% HPLC-grade methanol until chemical analysis. One tadpole per tub ($N = 192$ in total) was used for bufadienolide measurement, the other one was used to identify the developmental stage [34] by stereomicroscopic examination. The rest of the tadpoles were kept alive as part of another experiment (Z Mikó, D Holly, V Bókony, A Hettyey 2015, unpublished data).

(c) HPLC analysis

The protocol of our chemical analysis has been described in detail earlier [19]. In short, we homogenized each tadpole and dried the samples under vacuum to measure their dry mass. We re-dissolved the samples in 1 ml HPLC-grade absolute methanol, and filtered them using nylon syringe filters. We applied high-performance liquid chromatography with diode-array detection and mass spectrometry (HPLC-DAD-MS; LC-MS-2020, Shimadzu, Kyoto, Japan) to identify and quantify bufadienolide compounds in each sample [19]. Bufadienolides were recognized by their characteristic UV spectrum, and identified by comparing their peak retention time and m/z (mass-to-charge ratio) values to those of commercial standards and to the peaks present in a toxin sample obtained from the parotoid glands of juvenile common toads. We used the calibration curve of the bufotalin standard to express the bufotalin-equivalent concentration of each bufadienolide compound per sample; these values were then divided by tadpole dry mass to obtain concentrations per tadpole mass (ng mg^{-1}; [19,37,38]). Henceforth we refer to this variable as bufadienolide content. We did not statistically analyse toxin composition because it showed little variation: each tadpole contained six or seven out of the seven bufadienolide compounds detected in the laboratory experiment (except for one individual that contained only five) and 11 or 12 out of the 12 compounds detected in the mesocosm experiment (electronic supplementary material, table S2).

(d) Statistical analysis

All statistical analyses were run with R v. 3.3.1 [38], using the packages 'nlme' and 'lsmeans'. We analysed the effect of GBH treatment on bufadienolide content by linear mixed-effects (LME) models. The requirements of LME analysis were checked by inspecting residual plots; bufadienolide content was $\log_{10}$-transformed to improve model fit. We detected heteroscedasticity across treatment groups in the data of the laboratory experiment, so in these analyses we used the 'varIdent' function to estimate within-group variance for each group.

As recommended [40], we first tested which random-effects structure fitted our data best (electronic supplementary material, table S3), then we tested the fixed effect of the GBH treatment. For the laboratory experiment, the fixed factor was GBH treatment consisting of 13 treatment groups; we compared each of the 12 GBH treatments (i.e. low and high concentration combined with six different exposure times) to the control group by post hoc tests. For the mesocosm experiment, the fixed factor was GBH treatment consisting of three treatment groups; we compared both GBH treatments (i.e. low and high concentration) to the control group by post hoc tests. For the mesocosm experiment, we also checked whether the minor variation among tadpoles in developmental stage had any effect on bufadienolide content, by adding developmental stage as a second fixed factor into the model. To test whether the effect of GBH treatment differed between tadpoles originating from different ponds, we included pond as a fixed (instead of random) factor and tested its interaction with GBH treatment. In all analyses, the overall effect of each fixed factor or interaction was tested in analysis-of-variance tables with type-3 sums-of-squares (i.e. F-test for the proportion of variance explained by the factor or interaction), whereas post hoc tests were performed by calculating linear contrasts and correcting the p-values for multiple testing with Dunnett's method. Our analyses can be reproduced from the electronic supplementary material, table S4.

3. Results

There was significant variance among the GBH treatment groups both in the laboratory experiment (LME, $F_{13,48} = 82.22$, $p < 0.001$) and in the mesocosm experiment (LME, $F_{2,174} = 39.71$, $p < 0.001$). Post hoc tests showed that tadpoles in the laboratory exposed to the higher concentration of GBH for the entire duration of their larval development had significantly higher bufadienolide content than the control tadpoles (table 1 and figure 1); no other treatment group differed significantly from the control group (table 1). In the mesocosms, tadpoles exposed to the lower or the higher concentration of GBH both had significantly higher bufadienolide content than control tadpoles (table 1 and figure 1). This effect of GBH treatment was similar across tadpoles originating from different ponds (electronic supplementary material, figure S3), as the pond × treatment interaction was not significant (ANOVA, $F_{30,144} = 0.85$, $p = 0.685$). Bufadienolide content did not correlate with developmental stage (Spearman rank-correlation: $r_s = -0.01$, $p = 0.925$, $N = 192$); including developmental stage into the LME model did not change the effect of GBH treatment (developmental stage: $F_{3,171} = 0.03$, $p = 0.992$, GBH treatment: $F_{2,171} = 38.38$, $p < 0.001$).

4. Discussion

Our study showed that chronic exposure to a GBH significantly increased the bufadienolide content of toad tadpoles. The effects were statistically large (Hedges' $d > 1$) and ecologically relevant, being comparable to bufadienolide increases in other toad species which were induced by predatory threat [37,38] and caused considerable mortality to predators [18]. Furthermore, the GBH effects we found were dose dependent

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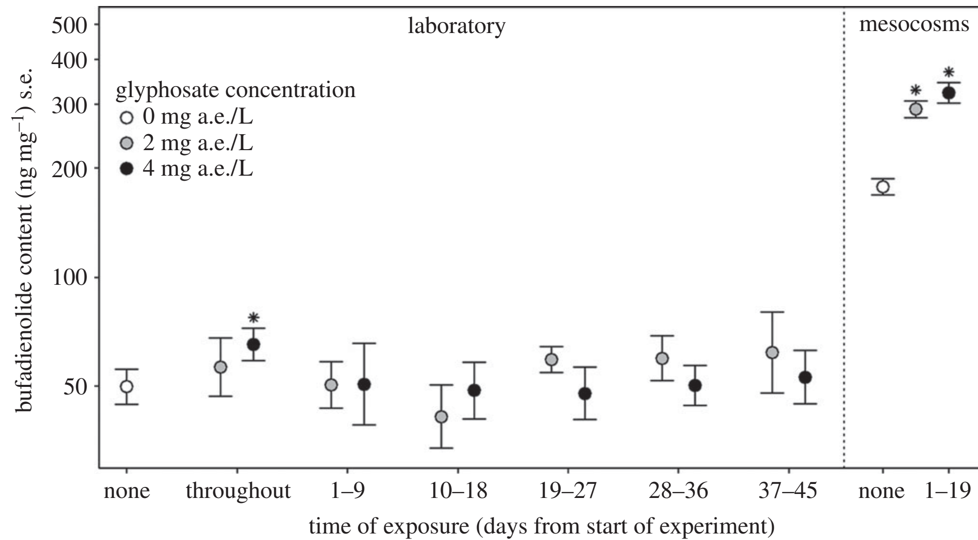

Figure 1. Bufadienolide content of toad tadpoles at the start of metamorphosis in the laboratory experiment and at developmental stage 34 in the mesocosm experiment. The groups marked with asterisks differ significantly ($p < 0.05$) from the control group. Note the logarithmic scale on the *Y*-axis.

Table 1. Dunnett's post hoc comparisons of bufadienolide content between the control group and each glyphosate-based herbicide treatment group in each experiment. Significant differences are highlighted in italics. Each difference was calculated as a linear contrast from a mixed-effects model (one model for each experiment). The proportional difference, calculated as $10^{\text{difference}}$ and converted to percentage, gives the unstandardized effect size (e.g. 174% means that the average bufadienolide content changed in response to the treatment to 174% of the control group's average). Hedges' *d* gives the standardized effect size ($d > 0.8$ is considered large, $d > 1$ is considered very large).

experiment	treatment group	difference ($\log_{10}$ ng mg^{-1}) ± s.e.	proportional difference (ng mg^{-1}) (%)	Hedges' *d*	d.f.	*t*	*p*-value
laboratory	2 mg a.e./L, period 1	0.005 ± 0.066	101	0.04	48	0.08	>0.999
	2 mg a.e./L, period 2	−0.083 ± 0.076	83	−0.47	48	−1.09	0.858
	2 mg a.e./L, period 3	0.076 ± 0.056	119	0.72	48	1.37	0.713
	2 mg a.e./L, period 4	0.079 ± 0.055	120	0.58	48	1.43	0.676
	2 mg a.e./L, period 5	0.095 ± 0.114	124	0.44	48	0.83	0.945
	2 mg a.e./L, throughout	0.055 ± 0.057	113	0.33	48	0.96	0.908
	4 mg a.e./L, period 1	0.007 ± 0.118	102	0.03	48	0.06	1.000
	4 mg a.e./L, period 2	−0.010 ± 0.064	98	−0.06	48	−0.16	1.000
	4 mg a.e./L, period 3	−0.019 ± 0.059	96	−0.12	48	−0.32	0.999
	4 mg a.e./L, period 4	0.004 ± 0.059	101	0.03	48	0.06	1.000
	4 mg a.e./L, period 5	0.027 ± 0.088	106	0.17	48	0.30	0.999
	4 mg a.e./L, throughout	*0.117 ± 0.039*	*131*	*1.02*	*48*	*3.02*	*0.037*
mesocosm	*2 mg a.e./L*	*0.196 ± 0.037*	*157*	*1.16*	*174*	*5.35*	*<0.001*
	4 mg a.e./L	*0.241 ± 0.037*	*174*	*1.27*	*174*	*6.58*	*<0.001*

and qualitatively consistent between two experiments that differed in several aspects, including the venue, the origin of the animals, the age of the tadpoles at toxin sampling, and the year of the study. Altogether these aspects make our finding robust [41,42]. This novel result that GBH exposure had a stimulating effect on the production of chemical defences is surprising, given the manifold negative effects of GBHs demonstrated so far on various fitness components in non-target organisms [26–29]. Because bufadienolides can provide protection for tadpoles from a variety of natural enemies [6,19], our finding adds to the emerging picture that the effects of GBHs in particular, and chemical pollutants in general, can have complex effects in natural systems [3,9]. For example, the GBH-increased bufadienolide content may reduce the threat posed by predators that are sensitive to these toxins, such as fish and newts [17], while the pesticide's negative effects on growth and development [29] may make the tadpoles more vulnerable to predators that are not deterred by bufadienolides, such as many invertebrates [17]. Furthermore, elevated toxin production might carry physiological costs, although the costs of bufadienolide synthesis and/or storage are not well understood yet [19,38,43].

The effect of GBH was stronger in the outdoor mesocosms than in the laboratory, which may be explained by several

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differences between the two experiments. Firstly, the tadpoles' age at toxin sampling is a probable source of variation because the bufadienolide content of common toad tadpoles drops shortly before metamorphosis, when the laboratory samples were taken, which may have left less room for responsiveness to environmental stress during this time [36]. Secondly, the effect of GBH may have been increased in mesocosms by the presence of additional stressors [4], including UV radiation, variation in temperature, pathogens present in pond water, or competition for food, which have been shown to exacerbate the lethal and sublethal effects of pesticides [28]. Thirdly, it is possible that spinach, the food we fed to tadpoles in the laboratory, is a poorer source for bufadienolide production (e.g. due to the hypocholesterolemic effect of its saponin content, [44]) than the diverse planktonic and epiphytic flora growing in outdoor mesocosms. Finally, population differences may have contributed to the lower sensitivity to GBH in the laboratory, although in the mesocosm experiment we found little variation among 16 populations in the effect of chronic GBH exposure on bufadienolide content, despite significant among-population heterogeneity in average toxin levels.

There are several alternative ways by which GBHs could influence the production of bufadienolides. One possibility is that elevated toxin levels result from a general response to physiological stress, given that they are expected to provide protection against a variety of stressors, including salinity, predators, pathogens, parasites, and competitors [6,19]. In line with this idea, our field observations [19] as well as a laboratory experiment [36] suggested that toad tadpoles respond to increased competition for food by producing more bufadienolides. Although we found no effect of predation risk in the latter two studies, challenging the idea that toad tadpoles would indiscriminately respond to any stressor by increased chemical defence, experiments with two other toad species found a positive effect of predation risk on some aspects of bufadienolide defences [37,38]. Another possibility is that GBHs inhibit the tadpoles' detoxification processes, thereby leading to the accumulation of bufadienolides. A study on stage 36–38 tadpoles of a toad species (*Rhinella arenarum*) reported that various GBH formulations decreased the activity of several esterase enzymes involved in detoxification [45], whereas in human liver cells glyphosate inhibited the activity of major xenobiotic-metabolizing enzymes of the cytochrome P450 family [46]. However, the role of these enzymes in bufadienolide metabolism is not known, and in general very few data exist on how toxin-producing amphibians deal with autotoxicity [47,48]. Finally, it is also possible that GBHs specifically increase the synthesis of bufadienolides. In animals, these toxins are synthesized from cholesterol by a chemical pathway that produces hydroxycholanates, i.e. bile acids [14,49]. GBHs might directly affect the bile acid pathway, for example, by upregulating the enzyme (CYP27) that controls the first step of the pathway. One of the transcriptional regulators of this enzyme is retinoic acid [50]; interestingly, a GBH (identical in composition to the formulation we used in our experiment) was found to increase endogenous retinoic acid activity in *Xenopus laevis* embryos [51]. GBHs might also affect the bile acid pathway indirectly, because cholesterol is also the precursor for the steroidogenic pathway that produces sex steroids and corticosteroids [14], and the enzymes involved in steroid biosynthesis are known targets for the actions of various endocrine-disrupting chemicals [52], including GBHs [53,54]. By inhibiting the steroidogenic pathway, GBHs might increase the availability of cholesterol and thereby facilitate the bile acid pathway that produces bufadienolides. More specific speculations are not possible at our current level of knowledge, because in amphibians the steps and regulators of bufadienolide synthesis are poorly known [49] and very few studies have been published on the endocrine-disrupting effects of GBHs [55,56].

It remains to be investigated whether the pattern observed in our study represents a general response of bufadienolide synthesis to GBHs and perhaps also to other endocrine-disrupting chemicals. If it does, our results indicate that pesticide pollution might exacerbate the problem of invasive toxic species. For example, in Australia, the survival of native tadpoles is reduced by poisoning from ingestion of toxic cane toad eggs, and predators suffer drastic mortality due to ingesting or mouthing cane toads [22]. As cane toads occupy a wide range of habitats and prefer anthropogenically altered sites [22], they may often come into contact with various pollutants and pesticides, which might contribute to the spatial heterogeneity in their toxicity [57]. Furthermore, increased toxicity of native species may also have far-reaching consequences for animal communities, for example, by driving their predators to switch to more palatable prey [17]. Therefore, we urge further studies to uncover how environmental contaminants affect chemical defences in general and bufadienolides in particular.

Ethics. All experimental procedures were carried out according to the permits issued by the Közép-Duna-Völgyi KTVF (KTVF: 603-3/2014, KTF: 2771-3/2015) and the Government Agency of Pest County, Hungary (PEI/001/389-4/2013). The experiments were further approved by the Ethical Commission of MTA ATK NÖVI.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors' contributions. A.H., Z.M., and V.B. designed the experiments; Z.M. performed the experiments; Á.M.M. and D.K. performed the HPLC analyses; V.B. conducted the statistical analyses and wrote the manuscript with substantial contributions from A.H., Z.M., and Á.M.M. All authors gave final approval for publication.

Competing interests. We have no competing interests.

Funding. Funding was provided by the 'Lendület' programme of the Hungarian Academy of Sciences (MTA, LP2012-24/2012), an FP7 Marie Curie Career Integration grant no. (PCIG13-GA-2013-631722) and the National Research, Development and Innovation Office (NKFIH) of Hungary (grant no. 115402). V.B. was supported by the János Bolyai Scholarship of the Hungarian Academy of Sciences.

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