THIS IS THE FINAL, ACCEPTED VERSION OF THE MANUSCRIPT

doi: 10.1098/rspb.2017.0493

published in

Proceedings of the Royal Society B – Biological Sciences 284: 20170493

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

Veronika Bókony[1*], Zsanett Mikó[1], Ágnes M. Móricz[2], Dániel Krüzselyi[2], Attila Hettyey[1]

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

[2] *Department of Pathophysiology, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman Ottó út 15, Budapest, 1022, Hungary*

[*]Author for correspondence (bokony.veronika@agrar.mta.hu).

Abstract

Chemical pollutants can exert various sub-lethal 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 defense 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 3-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.

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

Introduction

We live in an era of environmental pollution, with a broad array of chemical contaminants such as pesticides, heavy metals, and road deicers being introduced into the environment in ever-growing quantities. Besides causing mortality events, these contaminants can also exert a variety of sub-lethal 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 behaviors 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 defense. 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 defenses may be disrupted by chemical contaminants, although the effect is sometimes, counter-intuitively, positive [3]. Among vertebrates, chemical defense is most widespread in amphibians, a group of serious conservation concern due to their ongoing population declines worldwide [7]. In recent years, amphibian chemical defenses 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 to the defensive role of antimicrobial skin peptides against chytridiomycosis, a spreading lethal disease caused by the fungus *Batrachochytrium dendrobatidis*, and the effects of contaminants on this defense [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 signaling [14], and due to their cardiotonic effect, they are utilized as chemical defense 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 defenses from early on during their ontogeny [18,19] and show very little anti-predatory defenses in terms of morphology and behavior 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 defenses 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 defense.

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 sub-lethal 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 defense. 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 setup, in which we investigated the effect of chronic GBH exposure in tadpoles originating from several different populations, because both chemical defense [19] and susceptibility to pesticides [31,32] can vary across populations.

Methods

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.

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 (Mikó, Ujszegi, Gál & Hettyey, unpublished data). We did not measure the actual concentrations in the experimental containers in the present study, but in our earlier work using the same protocols we measured 1.41 ± 0.34 (SE) 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 were found in runoff after GBH use [23]. The concentrations given throughout the text henceforward are nominal.

Laboratory experiment

On the 28th of 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 12:12 h light:dark 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 [33] 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 4 replicates in each treatment by family combination (i.e. 4 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 SE) or only for a 9-days 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-days period, at the onset of metamorphosis (developmental stage 42, [33]). We randomly selected 5 individuals from each treatment group (one from each of 5 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].

Mesocosm experiment

Between 7 and 13 April 2015, we collected 40 eggs from each of 8 freshly laid clutches from each of 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 by family in 3-L containers holding 1 L of RSW at 20 °C and a 12:12 h light:dark 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 provides nutrients and refuges for tadpoles [30,34]. 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 two 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 4 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 SD: 33.76 ± 0.85). 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 in this stage [35]. 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 [33] by stereomicroscopic examination. The rest of the tadpoles were kept alive as part of another experiment (unpublished data).

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 in 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; [19,36,37]). Henceforth we refer to this variable as bufadienolide content. We did not statistically analyze toxin composition because it showed little variation: each tadpole contained 6 or 7 out of the 7 bufadienolide compounds detected in the laboratory experiment (except for one individual that contained only 5) and 11 or 12 out of the 12 compounds detected in the mesocosm experiment (electronic supplementary material, table S2).

Statistical analysis

All statistical analyses were run with R 3.3.1 [38], using the packages 'nlme' and 'lsmeans'. We analyzed 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 [39], 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 6 different exposure times) to the control group by post-hoc

tests. For the mesocosm experiment, the fixed factor was GBH treatment consisting of 3 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 done 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).

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, 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, 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: $F_{2,171} = 38.38$, $p < 0.001$).

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 [36,37] and caused considerable mortality to predators [18]. Furthermore, the GBH effects we found were dose-dependent 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 [40,41]. This novel result that GBH exposure had a stimulating effect on the production of chemical defenses 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 [19,37,42].

The effect of GBH was stronger in the outdoor mesocosms than in the laboratory, which may be explained by several 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 lab samples were taken, which may have left less room for responsiveness to environmental stress during this time [35]. 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 sub-lethal 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, [43]) 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 [35] 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 defense, experiments with two other toad species found a positive effect of predation risk on some aspects of bufadienolide defenses [36,37]. 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 [44], whereas in human liver cells glyphosate inhibited the activity of major xenobiotic-metabolizing enzymes of the cytochrome P450 family [45]. 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 [46,47]. 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 [14,48]. 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 [49]; 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 [50]. 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 [51], including GBHs [52,53]. 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 [48] and very few studies have been done on the endocrine-disrupting effects of GBHs [54,55].

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 [56]. 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 defenses in general and bufadienolides in particular.

Data Accessibility. The datasets supporting this article have been uploaded as part of the Supplementary Material.

Authors' Contributions. AH, ZsM and VB designed the experiments, ZsM performed the experiments, ÁMM and DK performed the HPLC analyses, VB conducted the statistical analyses. VB wrote the manuscript with substantial contributions from AH, ZsM, and ÁMM. 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 (PCIG13-GA-2013-631722), and the National Research, Development and Innovation Office (NKFIH) of Hungary (grant no. 115402). VB was supported by the János Bolyai Scholarship of the Hungarian Academy of Sciences.

References

1. Lürling M, Scheffer M. 2007 Info-disruption: pollution and the transfer of chemical information between organisms. *Trends Ecol. Evol.* **22**, 374–379. (doi:10.1016/j.tree.2007.04.002)
2. Köhler H-R, Triebskorn R. 2013 Wildlife ecotoxicology of pesticides: can we track effects to the population level and beyond? *Science* **341**, 759–765. (doi:10.1126/science.1237591)
3. Boyd RS. 2010 Heavy metal pollutants and chemical ecology: Exploring new frontiers. *J. Chem. Ecol.* **36**, 46–58. (doi:10.1007/s10886-009-9730-5)
4. Relyea RA, Mills N. 2001 Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). *Proc. Natl. Acad. Sci. U. S. A.* **98**, 2491–2496. (doi:10.1073/pnas.031076198)
5. Brodie ED. 2009 Toxins and venoms. *Curr. Biol.* **19**, R931–R935. (doi:10.1016/B978-0-12-415813-9.00014-3)
6. Hettyey A, Tóth Z, Van Buskirk J. 2014 Inducible chemical defences in animals. *Oikos* **123**, 1025–1028. (doi:10.1111/OIK.01338)
7. Campbell Grant EH et al. 2016 Quantitative evidence for the effects of multiple drivers

on continental-scale amphibian declines. *Sci. Rep.* **6**, 25625. (doi:10.1038/srep25625)

8. Davidson C, Benard MF, Shaffer HB, Parker JM, O'Leary C, Conlon JM, Rollins-Smith LA. 2007 Effects of chytrid and carbaryl exposure on survival, growth and skin peptide defenses in foothill yellow-legged frogs. *Environ. Sci. Technol.* **41**, 1771–1776. (doi:10.1021/es0611947)
9. Gahl MK, Pauli BD, Houlahan JE. 2011 Effects of chytrid fungus and a glyphosate-based herbicide on survival and growth of wood frogs (*Lithobates sylvaticus*). *Ecol. Appl.* **21**, 2521–2529.
10. Gibble RE, Baer KN. 2011 Effects of atrazine, agricultural runoff, and selected effluents on antimicrobial activity of skin peptides in *Xenopus laevis*. *Ecotoxicol. Environ. Saf.* **74**, 593–599. (doi:10.1016/j.ecoenv.2010.11.009)
11. Cary TL, Ortiz-Santaliestra ME, Karasov WH. 2014 Immunomodulation in post-metamorphic northern leopard frogs, *Lithobates pipiens*, following larval exposure to polybrominated diphenyl ether. *Environ. Sci. Technol.* **48**, 5910–5919. (doi:10.1021/es405776m)
12. Buck JC, Hua J, Brogan WR, Dang TD, Urbina J, Bendis RJ, Stoler AB, Blaustein AR, Relyea RA. 2015 Effects of pesticide mixtures on host-pathogen dynamics of the amphibian chytrid fungus. *PLoS One* **10**, 1–17. (doi:10.5061/dryad.45595)
13. Steyn PS, van Heerden FR. 1998 Bufadienolides of plant and animal origin. *Nat. Prod. Rep.* **15**, 397–413. (doi:10.1039/a815397y)
14. Fedorova O V., Zernetkina VI, Shilova VY, Grigorova YN, Juhasz O, Wei W, Marshall CA, Lakatta EG, Bagrov AY. 2015 Synthesis of an endogenous steroidal Na pump inhibitor marinobufagenin, implicated in human cardiovascular diseases, is initiated by CYP27A1 via bile acid pathway. *Circ. Cardiovasc. Genet.* **8**, 736–745. (doi:10.1161/CIRCGENETICS.115.001217)

15. Cunha Filho GA et al. 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. (doi:10.1016/j.toxicon.2005.01.017)

16. Tempone AG, Pimenta DC, Lebrun I, Sartorelli P, Taniwaki NN, de Andrade HF, Antoniazzi MM, Jared C. 2008 Antileishmanial and antitrypanosomal activity of bufadienolides isolated from the toad *Rhinella jimi* parotoid macrogland secretion. *Toxicon* **52**, 13–21. (doi:10.1016/j.toxicon.2008.05.008)

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

18. Hayes RA, Crossland MR, Hagman M, Capon RJ, Shine R. 2009 Ontogenetic variation in the chemical defenses of cane toads (*Bufo marinus*): Toxin profiles and effects on predators. *J. Chem. Ecol.* **35**, 391–399. (doi:10.1007/s10886-009-9608-6)

19. Bókony V et al. 2016 Variation in chemical defense among natural populations of common toad, *Bufo bufo*, tadpoles: the role of environmental factors. *J. Chem. Ecol.* **42**, 329–338. (doi:10.1007/s10886-016-0690-2)

20. 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. (doi:10.1007/s10750-006-0475-7)

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. (doi:10.1086/340599)

22. Shine R. 2010 The ecological impact of invasive cane toads (*Bufo marinus*) in Australia. *Q. Rev. Biol.* **85**, 253–291. (doi:10.1086/655116)

23. Edwards WM, Triplett GB. J, Kramer RM. 1980 A watershed study of glyphosate

transport in runoff. *J. Environ. Qual.* **9**, 661–665.

24. Govindarajulu PP. 2008 Literature review of impacts of glyphosate herbicide on amphibians: What risks can the silvicultural use of this herbicide pose for amphibians in B. C.? Report, *Minist. Environ. Victoria, BC.*

25. Mörtl M, Németh G, Juracsek J, Darvas B, Kamp L, Rubio F, Székács A. 2013 Determination of glyphosate residues in Hungarian water samples by immunoassay. *Microchem. J.* **107**, 143–151. (doi:10.1016/j.microc.2012.05.021)

26. Giesy J, Dobson S, Giesy JP, Dobson S, Solomon K, Solomon KR. 2000 Ecotoxicological risk assessment for Roundup herbicide. *Rev. Environ. Contam. Toxicol.* **167**, 35–120. (doi:10.1007/978-1-4612-1156-3_2)

27. Székács A, Darvas B. 2012 Forty years with glyphosate. In *Properties, synthesis and control of weeds* (ed Hasaneen MN), pp. 247–284. InTech. (doi:10.5772/52807)

28. Wagner N, Reichenbecher W, Teichmann H, Tappeser B, Lötters S. 2013 Questions concerning the potential impact of glyphosate-based herbicides on amphibians. *Environ. Toxicol. Chem.* **32**, 1688–1700. (doi:10.1002/etc.2268)

29. Mikó Z, Ujszegi J, Hettyey A. 2017 Age-dependent changes in sensitivity to a glyphosate-based pesticide in tadpoles of the common toad (*Bufo bufo*). *Aquat. Toxicol.* **187**, 48–54. (doi:10.1016/j.aquatox.2017.03.016)

30. Mikó Z, Ujszegi J, Gál Z, Imrei Z, Hettyey A. 2015 Choice of experimental venue matters in ecotoxicology studies: Comparison of a laboratory-based and an outdoor mesocosm experiment. *Aquat. Toxicol.* **167**, 20–30. (doi:10.1016/j.aquatox.2015.07.014)

31. Bridges CM, Semlitsch RD. 2000 Variation in pesticide tolerance of tadpoles among and within species of ranidae and patterns of amphibian decline. *Conserv. Biol.* **14**, 1490–1499. (doi:10.1046/j.1523-1739.2000.99343.x)

32. Cothran RD, Brown JM, Relyea RA. 2013 Proximity to agriculture is correlated with pesticide tolerance: Evidence for the evolution of amphibian resistance to modern pesticides. *Evol. Appl.* **6**, 832–841. (doi:10.1111/eva.12069)

33. Gosner KL. 1960 A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**, 183–190. (doi:10.2307/3890061)

34. Hettyey A, Tóth Z, Thonhauser KE, Frommen JG, Penn DJ, 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. (doi:10.1007/s00442-015-3382-7)

35. Üveges B, Fera G, Móricz ÁM, Krüzselyi D, Bókony V, Hettyey A. 2017 Age- and environment-dependent changes in chemical defences of larval and post-metamorphic toads. *BMC Evol. Biol.* (doi:10.1186/s12862-017-0956-5)

36. 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. (doi:10.1111/j.1365-2435.2008.01470.x)

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

38. R Core Team. 2016 R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

39. Zuur AF, Ieno EN, J.Walker N, Saveliev AA, Smith GM. 2009 *Mixed effects models and extensions in ecology with R*. Springer.

40. Bradford-Hill A. 1965 The enviroment and disease: association or causation? *Proc. R. Soc. Med.* **58**, 295–300. (doi:DOI: 10.1016/j.tourman.2009.12.005)

41. Richter SH, Garner JP, Würbel H. 2009 Environmental standardization: cure or cause

of poor reproducibility in animal experiments? *Nat. Methods* **6**, 257–61. (doi:10.1038/nmeth.1312)

42. Kurali A, Pásztor K, Hettyey A, Tóth Z. 2016 Toxin depletion has no effect on antipredator responses in common toad (*Bufo bufo*) tadpoles. *Biol. J. Linn. Soc.* **119**, 1000–1010. (doi:10.1111/bij.12864)

43. Price KR, Johnson IT, Fenwick GR. 1987 The chemistry and biological significance of saponins in foods and feedingstuffs. *Crit. Rev. Food Sci. Nutr.* **26**, 27–135. (doi:10.1080/10408397609527208)

44. Lajmanovich RC, Attademo AM, Peltzer PM, Junges CM, Cabagna MC. 2011 Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) tadpoles: B-esterases and glutathione S-transferase inhibitors. *Arch. Environ. Contam. Toxicol.* **60**, 681–689. (doi:10.1007/s00244-010-9578-2)

45. Abass K, Turpeinen M, Pelkonen O. 2009 An evaluation of the cytochrome P450 inhibition potential of selected pesticides in human hepatic microsomes. *J. Environ. Sci. Health. B.* **44**, 553–563. (doi:10.1080/03601230902997766)

46. Saporito RA, Donnelly MA, Spande TF, Garraffo HM. 2012 A review of chemical ecology in poison frogs. *Chemoecology* **22**, 159–168. (doi:10.1007/s00049-011-0088-0)

47. Moore DJ, Halliday DCT, Rowell DM, Robinson AJ, Keogh JS. 2009 Positive Darwinian selection results in resistance to cardioactive toxins in true toads (Anura: Bufonidae). *Biol. Lett.* **5**, 513–6. (doi:10.1098/rsbl.2009.0281)

48. Porto AM, Gros EG. 1971 Biosynthesis of the bufadienolide marinobufagin in toads *Bufo paracnemis* from cholesterol-20-14C. *Experientia* **27**, 506. (doi:10.1007/BF02147562)

49. Szanto A et al. 2004 Transcriptional regulation of human CYP27 integrates retinoid ,

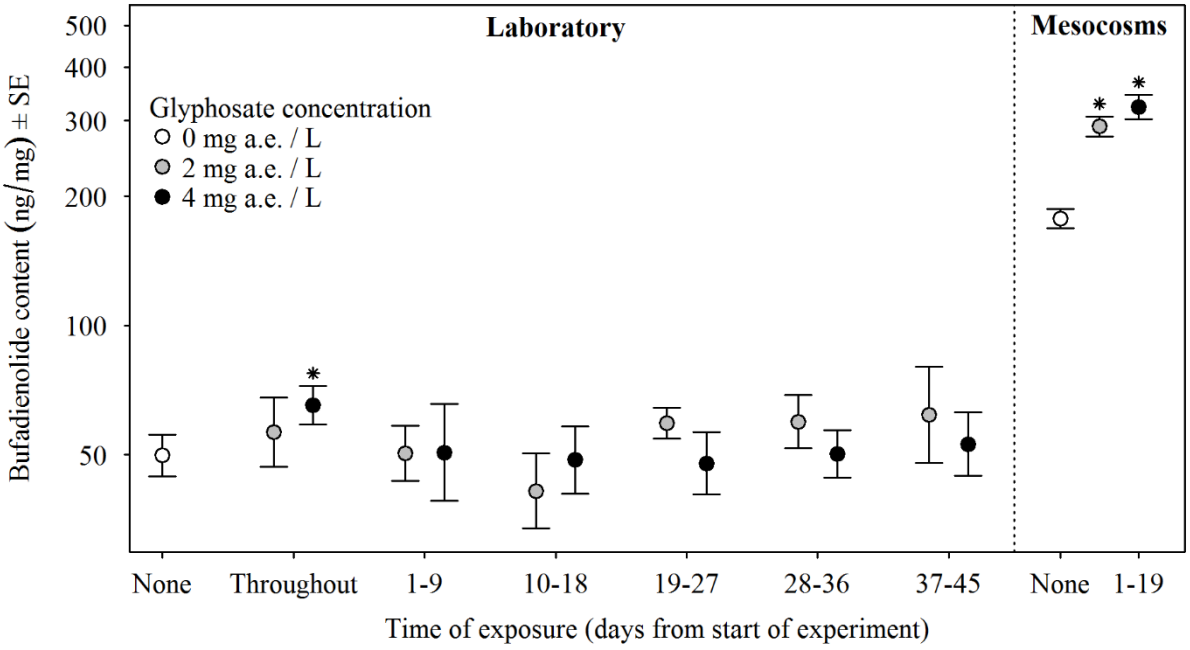
peroxisome proliferator-activated receptor , and liver X receptor signaling in macrophages. *Mol. Cell. Biol.* **24**, 8154–8166. (doi:10.1128/MCB.24.18.8154)

50. Paganelli A, Gnazzo V, Acosta H, López SL, Carrasco AE. 2010 Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. *Chem. Res. Toxicol.* **23**, 1586–1595. (doi:10.1021/tx1001749)
51. Sanderson JT. 2006 The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. *Toxicol. Sci.* **94**, 3–21. (doi:10.1093/toxsci/kfl051)
52. Richard S, Moslemi S, Sipahutar H, Benachour N, Seralini GE. 2005 Differential effects of glyphosate and roundup on human placental cells and aromatase. *Environ. Health Perspect.* **113**, 716–720. (doi:10.1289/ehp.7728)
53. Walsh LP, McCormick C, Martin C, Stocco DM. 2000 Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. *Environ. Health Perspect.* **108**, 769–776. (doi:10.1289/ehp.00108769)
54. Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004 Toxicity of glyphosate-based pesticides to four North American frog species. *Environ. Toxicol. Chem.* **23**, 1928. (doi:10.1897/03-71)
55. Lanctôt C, Navarro-Martín L, Robertson C, Park B, Jackman P, Pauli BD, Trudeau VL. 2014 Effects of glyphosate-based herbicides on survival, development, growth and sex ratios of wood frog (*Lithobates sylvaticus*) tadpoles. II: Agriculturally relevant exposures to Roundup WeatherMax® and Vision® under laboratory conditions. *Aquat. Toxicol.* **154**, 291–303. (doi:10.1016/j.aquatox.2014.05.025)
56. Phillips BL, Shine R. 2006 Spatial and temporal variation in the morphology (and thus, predicted impact) of an invasive species in Australia. *Ecography* **29**, 205–212. (doi:10.1111/j.2006.0906-7590.04413.x)

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 bold. 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 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) ± SE	Proportional difference (ng/mg)	Hedges' *d*	*d.f.*	*t*	*p*
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**

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.



Supplementary material for

Veronika Bókony, Zsanett Mikó, Ágnes M. Móricz, Dániel Krüzselyi, Attila Hettyey (2017):

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

Proceedings of the Royal Society B – Biological Sciences

doi: 10.1098/rspb.2017.0493

This document (**Supplement 1**: Additional tables and figures) contains the following items:

Table S1: Geographical coordinates of the 16 ponds used for toad egg collection in the mesocosm experiment

Table S2: Bufadienolide compounds found in toad tadpoles, and the percentage of tadpoles in which each compound occurred in each experiment

Table S3: AIC values of LME models with various random-effects structures

Figure S1: Bufadienolide content of toad tadpoles originating from different ponds in the mesocosm experiment

Further supplementary material available as separate file:

Supplement 2: Data analysed (Table S4).

Table S1. Geographical coordinates of the 16 ponds used for toad egg collection in the mesocosm experiment.

Pond	Latitude (°N)	Longitude (°E)	Altitude (m)
Babatpuszta	47.6253	19.37756	171
Bajdázói-tó	47.90327	18.97845	291
Bika-tó	47.60264	19.52324	145
Domonyvölgy	47.63509	19.40382	156
Ebszőnybánya	47.70253	18.64766	122
Határréti-tó	47.6466	18.90853	168
Hegyes-kő alatti tó	47.71032	18.66598	186
János-tó	47.71407	19.01978	408
Letkési halastó	47.87162	18.78092	105
Nagybörzsönyi halastó	47.93143	18.81972	195
Perőcsényi halastó	47.98632	18.84272	150
Pomázi-sík	47.65987	19.04651	123
Smanyina	47.70002	18.88652	407
Szentkirály	47.50091	19.46551	166
Törökmező	47.83461	18.94117	180
Úri halastó	47.38953	19.57526	122

Table S2. Bufadienolide compounds found in toad tadpoles, and the percentage of tadpoles in which each compound occurred in each experiment.

Retention time (min)	Mass-to-charge ratio (m/z)	% occurrence in laboratory	% occurrence in mesocosms
2.7	419	100%	–
4.3	403	54%	–
4.5	701	–	100%
5.7	715	–	100%
7.0	729	100%	100%
7.4	715	–	100%
8.1	727	98%	100%
9.7	729	–	100%
10.1	701	–	88%
11.4	715	100%	100%
13.0	713	100%	100%
13.5	743	–	100%
14.2	715	–	99%
17.7	757	100%	100%

Table S3. AIC values of LME models with various random-effects structures.

Random effects	Laboratory	Mesocosms
None	24.37	-53.79
Family	**16.07**	–
Pond	–	**-75.07**
Block	22.29	-51.79
Block nested in family	18.07	–
Block nested in pond	–	-73.32
Linear spatial correlation	–	-71.95
Gaussian spatial correlation	–	-66.99
Exponential spatial correlation	–	-74.13
Spherical spatial correlation	–	-71.95
Rational quadratic spatial correlation	–	-74.17

We compared the fit of various models containing different random effects by Akaike's information criterion (AIC), and we chose the model with the lowest AIC value (highlighted in bold). The null model assumed no random effects. The two potential random effects were family and block for the laboratory data and pond and block for the mesocosm data. For each experiment, we compared the fit of the null model to the fit of two models each containing one of the two random factors, and a third model that contained both random factors (block nested in family or pond). For the mesocosm data we considered 5 further random structures that assumed that differences in bufadienolide content varied with the distance among ponds following a certain function (i.e. linear, Gaussian, exponential, spherical, or rational quadratic). All models included the effect of the GBH treatment of the respective experiment as fixed factor.

Figure S1. Bufadienolide content of toad tadpoles originating from different ponds in the mesocosm experiment.