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**Does the glucocorticoid stress response make toads more toxic? An experimental study
on the regulation of bufadienolide toxin synthesis**

Bálint Üveges^{1,2*}, Csenge Kalina^{1,3}, Krisztina Szabó⁴, Ágnes M. Móricz⁵, Dóra Holly¹, Caitlin R. Gabor^{1,6}, Attila Hettyey¹, Veronika Bókony^{1,3}

¹Department of Evolutionary Ecology, Plant Protection Institute, Centre for Agricultural Research, Eötvös Loránd Research Network, 1022, Herman Ottó út 15, Budapest, Hungary

²Molecular Ecology and Evolution at Bangor, School of Natural Sciences, Bangor University, Environment Centre Wales, LL57 2UW, Bangor, Wales, United Kingdom

³Department of Ecology, Institute of Biology, University of Veterinary Medicine, 1078, István u. 2, Budapest, Hungary

⁴Division of Clinical Immunology, Department for Internal Medicine, Faculty of Medicine, University of Debrecen, 4032, Móricz Zsigmond út 22, Debrecen, Hungary

⁵Department of Pathophysiology, Plant Protection Institute, Centre for Agricultural Research, Eötvös Loránd Research Network, 1022, Herman Ottó út 15, Budapest, Hungary

⁶Department of Biology, College of Science and Engineering, Texas State University, 601 University Dr., San Marcos, TX 78666, Texas, USA

***Corresponding author:** Bálint Üveges (email: uveges.balint@yahoo.de)

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Abstract

Chemical defence is a crucial component of fitness in many organisms, yet the physiological regulation of defensive toxin synthesis is poorly understood, especially in vertebrates.

Bufadienolides, the main defensive compounds of toads, are toxic to many predators and other natural enemies, and their synthesis can be upregulated by stressors including predation risk, high conspecific density, and pollutants. Thus, higher toxin content may be the consequence of a general endocrine stress response in toads. Therefore, we hypothesized that bufadienolide synthesis may be stimulated by elevated levels of corticosterone (CORT), the main glucocorticoid hormone of amphibians, or by upstream regulators that stimulate CORT production. To test these alternatives, we treated common toad tadpoles with exogenous CORT (exoCORT) or metyrapone (MTP, a CORT-synthesis inhibitor that stimulates upstream regulators of CORT by negative feedback) in the presence or absence of predation cues for 2 or 6 days, and subsequently measured their CORT release rates and bufadienolide content. We found that CORT release rates were elevated by exoCORT, and to a lesser extent also by MTP, regardless of treatment length. Bufadienolide content was significantly decreased by treatment with exoCORT for 6 days, but was unaffected by exposure to exoCORT for 2 days or to MTP for either 6 or 2 days. Presence or absence of predation cues affected neither CORT release rate nor bufadienolide content. Our results suggest that changes in bufadienolide synthesis in response to environmental challenges are not driven by CORT but may rather be regulated by upstream hormones of the stress response.

Introduction

Using toxic or noxious chemical compounds for protection from natural enemies, such as predators, pathogens, parasites and competitors is widespread in the animal kingdom (Blum, 1981; Brodie, 2009; Casewell et al., 2013; Pawlik, 1993). Chemical defence is important from the perspective of ecology and life-history evolution, as chemically protected animals can have a longer life span (Hossie et al., 2013) and occupy a larger niche space (Arbuckle et al., 2013). Toxins can also influence survival not only of the defended animals themselves (Hayes et al., 2009; Llewelyn et al., 2012), but also of other species. For instance, when consuming unusually toxic prey, predators can suffer high mortality and population declines, as shown by the infamous example of invasive cane toads (*Rhinella marina*) that endanger native fauna (Shine, 2010). On the other hand, toxins can also change trophic interactions and community structure if predators learn to avoid the consumption of toxic animals and switch to alternative prey (Nelson et al., 2010; Nelson et al., 2011; Webb et al., 2008). Furthermore, the presence of effective toxins may also induce co-evolutionary arms races between prey that produce them and predators that adapt to their consumption by evolving toxin resistance (Brodie and Brodie, 1990; Ujvari et al., 2015). Despite the widespread occurrence of chemical defence and its importance for multiple aspects of biology, the physiological regulation of toxin synthesis remains poorly known in a plethora of species.

Toads (Bufonidae, Amphibia) produce skin secretions containing, among others, bufadienolides as defensive compounds, which can be effective against predators and possibly also against pathogens (Barnhart et al., 2017; Cunha Filho et al., 2005; Tempone et al., 2008; Toledo and Jared, 1995; Üveges et al., 2019). Bufadienolides are potent inhibitors of Na^+/K^+ -ATPase (Lingrel, 2010; Pierre and Xie, 2006; Schoner and Scheiner-Bobis, 2007), and can cause serious symptoms upon ingestion including nausea, convulsions, hypertension, cardiac arrhythmia and even death (Chen and Huang, 2013; Kamboj et al., 2013; Toledo and Jared,

1995). The rate of bufadienolide synthesis in toads can be increased by various environmental factors such as predation risk (Hettyey et al., 2019), high conspecific density (Bókony et al., 2018; Üveges et al., 2021), reduced food availability (Üveges et al., 2017) and anthropogenic pollutants (Bókony et al., 2017). This high diversity of inducing stressors raises the question if upregulated bufadienolide synthesis is a consequence or part of the generalized endocrine stress response.

The endocrine stress response, which is highly conserved across vertebrates (Denver, 2009; Godoy et al., 2018), serves to maintain homeostasis by allostasis and ensure survival by responding to environmental perturbation (Koolhaas et al., 2011; McEwen, 1998; McEwen and Wingfield, 2003). One integral part of this system is the hypothalamic–pituitary–adrenal (HPA, or hypothalamic–pituitary–interrenal, HPI) axis (Fig. 1). Perception of environmental stressors triggers the discharge of corticotropin-releasing factor (CRF) from the hypothalamus, which in turn induces production of adrenocorticotrophic hormone (ACTH) in the anterior pituitary gland. ACTH stimulates the adrenal glands (or interrenal glands in amphibians and fish, Denver, 2009) to produce glucocorticoid hormones (GCs, notably cortisol and corticosterone, CORT). GCs modulate a multitude of physiological processes including metabolism and immune response, and facilitate recovery after stress by promoting energy uptake of cells and tissues via gluconeogenesis (Denver, 2009; Godoy et al., 2018; McEwen, 1998; McEwen, 2004; McEwen and Wingfield, 2003; Sterling and Eyer, 1988). On the other hand, chronic stress might lead to long-term elevated levels of circulating GCs, resulting in adverse effects such as suppression of the immune system and cardiovascular, neurological and metabolic disorders (Denver, 2009; Godoy et al., 2018; McEwen, 1998; McEwen, 2004; McEwen and Wingfield, 2003; Sterling and Eyer, 1988). To keep GC levels in check, the HPA/HPI axis is under internal control by negative feedback loops. For instance,

high levels of circulating GCs inhibit the release of CRF and ACTH, thus returning GC secretion to baseline levels (Gjerstad et al., 2018; Godoy et al., 2018; Herman et al., 2020).

Various environmental stressors that trigger the GC response, such as predation risk (Berger et al., 2007; Middlemis Maher et al., 2013), intraspecific competition (Braasch et al., 2014; Eraud et al., 2008; Glennemeier and Denver, 2002a) and anthropogenic pollutants (Gabor et al., 2018; Monclús et al., 2018; Ye et al., 2015), also elevate the synthesis of bufadienolide toxins (Bókony et al., 2017; Bókony et al., 2018; Hettyey et al., 2019; Üveges et al., 2021; Üveges et al., 2017). Further similarities also suggest a physiological link between the two groups of compounds. Both CORT and bufadienolides are synthesized from the precursor cholesterol (Fedorova et al., 2015; Payne and Hales, 2004), and the synthesis of cardiotonic steroids, the compound family bufadienolides belong to, has been suggested to be upregulated by ACTH in mammals (Dostanic-Larson et al., 2005; Laredo et al., 1994; Laredo et al., 1995; Li et al., 1994; Sophocleous et al., 2003; Vinge et al., 1993; Yamada et al., 1997). However, the specific physiological regulatory mechanisms behind the synthesis of bufadienolides are still largely unknown (although see Dostanic-Larson et al., 2005; Fedorova et al., 1998), and explicit information about how GCs affect bufadienolide synthesis is, to our knowledge, entirely lacking.

Given the information presented above, we hypothesized that the HPI axis may provide a key to understanding the proximate mechanism of what makes toads more (or less) toxic. Specifically, we formulated two alternative hypotheses (Fig. 1). According to hypothesis 1 (H1), increased CORT levels upregulate bufadienolide synthesis directly. Conversely, hypothesis 2 (H2) postulates that bufadienolide production is regulated by upstream hormones of the HPI axis, which simultaneously increase the levels of both CORT and bufadienolides (Fig. 1). To test these alternatives, we reared tadpoles of the common toad (*Bufo bufo*) in the laboratory and exposed them to treatments with exogenous corticosterone

(exoCORT), metyrapone (MTP, a CORT synthesis inhibitor) or a solvent control. We predicted that treatment with exoCORT would lead to increased bufadienolide content if H1 was true, but to decreased bufadienolide content if H2 was true (due to negative feedback loops inhibiting expression of upstream regulators, Fig. 1). Metyrapone is used to test HPA functionality by blocking CORT synthesis, which in turn can stimulate the secretion of CRF and ACTH (Gjerstad et al., 2018; Godoy et al., 2018; Herman et al., 2020). Thus, in toad tadpoles treated with MTP, we expected to observe a decrease in bufadienolide content if H1 was true, but an increase if H2 is true (Fig. 1). Because earlier results indicated that MTP may not reduce CORT in the absence of stressors (Gabor et al., 2018), we combined the chemical treatments with a predator treatment (presence or absence of predation cues; Fig. 2) since predator presence is a natural stressor highly relevant to chemical defence. To discriminate between the acute and chronic effects of CORT on bufadienolide synthesis, we applied the treatments for either two or six days (Fig. 2).

Materials and methods

Experimental procedures

We collected 50 common toad eggs from each of 8 clutches (egg strings) in April 2019 from a pond located in the Pilis Mountains, Hungary (Zánkói rét, 47.740515° N, 19.016276° E). We transported eggs to the laboratory at the Plant Protection Institute in Budapest. Eggs and embryos from each clutch were kept together in 1 L reconstituted soft water (RSW, 48 mg/L NaHCO₃, 30 mg/L CaSO₄×2H₂O, 61 mg/L MgSO₄×7H₂O and 2 mg/L KCl dissolved in UV treated, reverse osmosis filtered, aerated tap water) in the laboratory until hatching, after which the water level was increased to 9 L (container size: 26.8 × 36.9 × 16.6 cm, width × length × height) until tadpoles reached the free-swimming stage (developmental stage 25, Gosner, 1960). Air temperature in the laboratory was 20.1 ± 1.1 °C resulting in 19.0 ± 0.2 °C

water temperature in tadpole containers. The light:dark cycle was adjusted weekly to simulate natural changes in photoperiod.

We began the experiment when the tadpoles reached the free-swimming stage (day 1). We haphazardly selected 36 toad tadpoles from each clutch and placed them individually into 2-L plastic boxes ($11.8 \times 16.5 \times 12.8$ cm) containing 1 L RSW. We fed experimental tadpoles throughout the study twice a week *ad libitum*. To ensure that cholesterol, the precursor for both endogenous CORT and bufadienolides, was not a limiting factor for the tadpoles, we fed them with a 20:1 mixture of slightly boiled, chopped spinach and minced axolotl food (JBL NovoLotl M, fat content 12 %). We changed the water of tadpoles twice a week for two weeks, until the start of the treatment period (see below).

On day 15 of the experiment, we assigned the tadpoles randomly to treatments with exogenous chemicals combined with the simultaneous presence or absence of predation cues for either 6 or 2 days in a fully crossed design (see below for details, Fig. 2). We applied treatments during the third week of the experiment, when toxin production of common toad tadpoles reacts strongest to environmental change (Ujszegi et al., 2017; Üveges et al., 2017). We applied treatments only for relatively short periods because CORT inhibits growth and development of tadpoles (Glennemeier and Denver, 2002b) and we wanted to avoid inducing a strong effect on the formation of cells producing bufadienolides (Delfino et al., 1995). Experimental containers were arranged in a randomised block design with each block containing one individual from each treatment \times clutch combination. We repeated each treatment \times clutch combination three times (3 blocks). Therefore, the total number of experimental units was 288 toad tadpoles (8 clutches \times 2 treatment lengths (6 or 2 days) \times 3 chemical treatments (exoCORT, MTP and control) \times 2 predation cue treatments (cues present or absent) \times 3 blocks). During the experiment, 3 tadpoles died for unknown reasons, reducing the final sample size to 285.

To manipulate the internal CORT levels of experimental tadpoles, we exposed them either to exoCORT or metyrapone (Sigma-Aldrich). As a solvent control treatment, the third group of the tadpoles received the same concentration of ethanol (EtOH) as their conspecifics in the exoCORT and MTP treatments. We did not use a control treatment with RSW only (without EtOH), because previous studies showed that the low concentration of ethanol used in our experiment does not affect the development and growth of tadpoles (Rohr et al., 2013; Young et al., 2020), including common toad larvae (Verebélyi, 2017). Right before the start of treatments, we created stock solutions of CORT (cc. 25 mg/ml) and MTP (cc. 30 mg/ml) by dissolving them in 96% EtOH and stored these in the dark at 4 °C until use. Similar to previous studies (Forsburg et al., 2019; Gabor et al., 2019; Glennemeier and Denver, 2002a; Glennemeier and Denver, 2002b), we diluted stock solutions to final concentrations of 125 nM exoCORT, 110 µM MTP, and 1 µl/L EtOH by adding them to RSW before each change of the rearing water of tadpoles. These concentrations of exoCORT and MTP increase and decrease, respectively, CORT in amphibian larvae, by approximately 50 % (Forsburg et al., 2019; Gabor et al., 2019; Glennemeier and Denver, 2002a; Glennemeier and Denver, 2002b). We exposed tadpoles to exogenous chemicals by changing the water of experimental animals using these freshly prepared solutions on days 15, 17 and 19 (6-days treatment) or only on day 19 (2-days treatment, Fig. 2). This way, tadpoles were able to absorb exoCORT and MTP from their rearing water (Gabor et al., 2018; Glennemeier and Denver, 2002a; Glennemeier and Denver, 2002b; Hayes and Wu, 1995; Middlemis Maher et al., 2013).

During the treatment period, half of the experimental tadpoles received cues indicating the presence of common perch (*Perca fluviatilis*), a predatory fish to which common toad larvae can respond with increased bufadienolide production (Hettyey et al., 2019). Predator cues were provided by six fish (body mass, mean \pm SD: 39.28 \pm 7.07 g) kept under laboratory conditions in 130 L aerated RSW, which was changed once a week. Fish were fed three times

a week with 40 live agile frog (*Rana dalmatina*) tadpoles and Tubifex worms *ad libitum* during the first two weeks of the experiment. From day 14 until termination of the experiment we fed fish daily with 6.06 ± 0.38 g (mean \pm SD) live agile frog tadpoles only. We fed fish with tadpoles to simulate a high risk of tadpole predation in general and to thereby elicit strong anti-predator responses in toad larvae (Hettyey et al., 2015; Laurila et al., 1997; Schoeppner and Relyea, 2005). We did not offer common toad tadpoles as food, because fishes often, but not always, avoid consuming them (Üveges et al., 2019), which would have made it difficult to obtain replicable quantities of chemical cues of predation risk. Agile frog tadpoles used for feeding fish were collected as eggs in a pond in the Pilis Mountains, Hungary (Jávortó, 47.713646° N, 19.019953° E). Agile frog tadpoles, and toad tadpoles used for the preparation of stimulus water (see below) were raised in the same way as focal tadpoles until developmental stage 25, after which they were reared in outdoor mesocosms. After termination of the experiment, all remaining tadpoles were released to their pond of origin.

During the treatment period, we created stimulus water daily by homogenizing 276.33 ± 27.82 g (mean \pm SD, days 15-18) and 487.55 ± 14.35 mg toad tadpoles (days 19-20) with a blender in ca. 50 ml RSW (Benard and Fordyce, 2003; Hettyey et al., 2019). This resulted in the immediate death of tadpoles. We opted for this method, because we did not want chemicals (e.g. MS222) used for euthanizing tadpoles to affect our experimental animals (Achtymichuk et al., 2022). We then added this homogenate to 2 and 3.5 L water, respectively, taken from the fish tank 2–3 hours after feeding the fish. This way, the concentration of chemical cues in the stimulus water was kept constant during the whole treatment period. We refilled the fish tank to the original volume with RSW after each stimulus-water preparation. We added the tadpole homogenate to ensure that experimental tadpoles were exposed to sufficiently high concentrations of conspecific prey-borne cues of

predation to elicit strong anti-predator responses (Hettyey et al., 2015; Laurila et al., 1997; Schoeppner and Relyea, 2005). During the treatment period we added 20 ml of stimulus water (predation cue treatment) or clean RSW (control treatment) to the water of focal tadpoles daily using a pipette. As a result, experimental tadpoles were exposed to chemical cues corresponding to 34.05 ± 1.15 mg/L fish (sources of kairomones, mean \pm SD), 0.89 ± 0.01 mg/L agile frog tadpoles (chemical cues released by the fish digesting frog tadpoles and sources of alarm pheromones or ‘Schreckstoff’, von Frisch, 1942), as well as to 2.66 ± 0.21 mg/L homogenized conspecifics (sources of chemical cues of conspecifics released by mechanical damage).

We terminated the experiment on day 21 after applying a non-invasive water-borne hormone sampling method which measures the amount of CORT released by tadpoles into the surrounding water (Baugh et al., 2018; Forsburg et al., 2019; Gabor et al., 2013a). This method provides a CORT measurement that is repeatable within individuals, correlates with plasma levels, and responds to ACTH challenge (Forsburg et al., 2019; Gabor et al., 2013a; Gabor et al., 2016). All experimental tadpoles were transferred from their rearing containers to individual 0.5 L plastic cups containing 100 ml RSW in a random order between 09:00 and 10:00 am. To avoid transferring exogenous chemicals, we first poured the rearing water of experimental animals through a stretched-out aquarium net. Then we used a dry piece of mosquito net (single-use for each animal) to transfer each tadpole one-by-one from the surface of the aquarium net into the plastic cups. After each tadpole spent 1 hour in the cup, we poured the water into individual polypropylene (PP) containers and stored the water samples at -20°C until analysis. We then preserved each tadpole in a microcentrifuge tube containing HPLC grade absolute methanol for chemical analysis of their toxin content, which resulted in the immediate death of tadpoles. Developmental stage of tadpoles (median: 35,

range: 32-37, Gosner, 1960) was determined by inspecting the preserved tadpoles under a stereomicroscope.

The study was approved by the Environment Protection and Nature Conservation Department of the Pest county Bureau of the Hungarian Government (PE-06/KTF/8060-1/2018, PE-06/KTF/8060-2/2018, PE-06/KTF/8060-3/2018, PE/EA/295-7/2018,) as well as by the Ethics Committee of the Plant Protection Institute, Centre for Agricultural Research, Eötvös Loránd Research Network.

Preparation of hormone samples and analysis of corticosterone release rates

We extracted the CORT content of water samples using C18 SPE columns (Sep-Pak Vac, 3cc/500mg, Waters Inc.) following an established protocol (Forsburg et al., 2019; Gabor et al., 2013a; Gabor et al., 2016). We primed each SPE column with 4 ml HPLC grade abs. methanol and 4 ml reverse-osmosis filtered water. Samples were forced through SPE columns using Tygon tubing under vacuum. We eluted samples with 4 ml HPLC grade absolute methanol into 5 ml PP microcentrifuge tubes. The eluted solution was kept at -20 °C until samples were transferred into 15 ml PP centrifuge tubes and dried out by vacuum centrifugation (40 °C, 1000 rpm, 100 Pa) and frozen again. Samples were analysed using a competitive enzyme-linked immunosorbent assay (ELISA, Cayman Chemical Inc.) following the manufacturers' protocol and based on previously published methodology (Gabor et al., 2013a; Gabor et al., 2016). We dissolved each sample in 12.5 µl of 96 % EtOH, vortexed them for 5 minutes, and subsequently added 237.5 µl ELISA buffer to obtain a final volume of 250 µl. Samples obtained this way were homogenised on an orbital shaker for 45 minutes to promote dissolution of CORT in the ethanol-buffer solution before transferring them to plates. Two samples were lost during the preparation process, leaving us with 283 samples to

be analysed for CORT content. The ELISA colour reaction was quantified at 412 nm wavelength using a microplate reader (Synergy HT, Bio-Tek Instruments).

Because we had no prior information on the range of waterborne CORT concentration in common toad samples, we prepared a dilution series from 6 samples to estimate the approximate range of sample concentrations in our study. To account for variation in the CORT concentrations of our samples, we then analysed two dilutions of each sample that would potentially fit in the linear region of the standard curve of the CORT standard. For subsequent statistical analyses, we used CORT concentrations of the dilution with the best fit. CORT concentrations were corrected for dilution. Due to limited ELISA kit availability, we could not analyse samples of the same dilution in duplicate. Therefore, intra-assay coefficient of variation (CV) could not be calculated. The inter-assay CV, calculated from concentrations of the CORT standard, was 9.4 %.

Preparation of toxin samples and analysis of bufadienolide content

We prepared and analysed bufadienolide samples following an established protocol (for a detailed description of methods see the Supplementary material and Hettyey et al., 2019; Üveges et al., 2021). Briefly, we homogenised preserved tadpoles and dried the resulting homogenates, then we measured dry mass of samples and re-dissolved them in 1 ml HPLC-grade absolute methanol. Finally, we filtered samples and analysed the resulting solution using high-performance liquid chromatography with diode-array detection and mass spectrometry (HPLC-DAD-MS). Bufadienolides were identified based on their characteristic peaks in the UV spectrum (Benard and Fordyce, 2003; Bókony et al., 2018; Hagman et al., 2009; Hettyey et al., 2019; Üveges et al., 2017; Üveges et al., 2019), by co-injection with selected bufadienolide standards and by additionally analysing a bulk toxin sample from 49 juvenile common toads.

Statistical analysis

We considered a specific bufadienolide to be present if its signal to noise ratio was at least three in the chromatogram (Hettyey et al., 2019; Üveges et al., 2021; Üveges et al., 2019). We estimated the quantity of each compound from the area values of MS chromatogram peaks based on the calibration curve of the marinobufotoxin standard. We subsequently summed these values to obtain estimates of total bufadienolide quantity (TBQ) for each individual. This approach results in rough estimates of bufadienolide content, but because commercially available standards are lacking for most bufadienolide compounds, this is currently the best quantification method available (Benard and Fordyce, 2003; Hagman et al., 2009; Üveges et al., 2021).

For data analysis, CORT release rate for each tadpole was expressed as the total amount of CORT released per hour, divided by tadpole dry mass (pg/mg/h). Toxin content was expressed as mass-corrected total bufadienolide quantity (mcTBQ, ng/mg) by dividing the total bufadienolide quantity by tadpole dry mass. The response variables were adjusted to body mass to account for the potential allometric relationship between body mass and TBQ or CORT release rate (Fig. S1), and because body mass and developmental stage were highly correlated (Fig. S2).

All statistical analyses were run in R 4.0 (R Development Core Team, 2017) using linear mixed-effects models. We log₁₀-transformed both CORT release rate and mcTBQ to meet the model assumptions based on diagnostic residual plots. We used the 'lmer' function of the 'lme4' package (Bates et al., 2015) to analyse CORT release rate. We included treatment length (2 or 6 days), presence or absence of predation cues, and the type of exogenous chemicals (exoCORT, MTP or EtOH) as fixed factors, and developmental stage centred to the mean as a covariate, as well as all two-way interactions between the fixed

factors and their three-way interaction. We included clutch and block as crossed random factors. To analyse \log_{10} -transformed bufadienolide content, we used the same model structure as for CORT release rate, except that clutch was used as the sole random factor. Preliminary analysis of data indicated that block had no effect on mcTBQ, therefore, it was omitted from this model. To account for differences in variances between the twelve treatment combinations and to improve model fit, we included the 'weights' argument with the 'varIdent' function in the mcTBQ model, which was run with the 'lme' function of the 'nlme' package (Pinheiro et al., 2017). We obtained *P* values from type-2 analysis-of-deviance (ANOVA) tables implemented in the 'car' package (Fox and Weisberg, 2019). We conducted post-hoc pairwise comparisons among treatment groups using linear contrasts implemented in the 'emmeans' package (Lenth et al., 2019). *P* values were corrected for multiple comparisons by using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995; Pike, 2011). We present the results of pairwise comparisons using means \pm 84% confidence intervals (CI), as suggested by Payton et al. (2003) for assessment of equality among groups (Fig. 3). Non-overlapping CIs indicate significant differences between groups after correction for false discovery rate. Additionally, we conducted Pearson's product-moment correlation implemented in base R and partial correlation controlling for developmental stage using the 'ppcor' package (Kim, 2015) between \log_{10} transformed CORT release rate and \log_{10} transformed mcTBQ.

Results

Corticosterone release rate of tadpoles was significantly affected by exogenous chemicals (Table 1). Tadpoles exposed to exoCORT released significantly more CORT compared to the control and MTP-treated groups, while tadpoles in the MTP group exhibited intermediate CORT release rates, differing significantly from those in the control and exoCORT groups (Table 2, Figure 3A). The effect of developmental stage on CORT release rate was also

significant: more developed tadpoles excreted lower amounts of CORT (Table 1, Fig. S3A). Treatment length, predation cues and their interactions had no significant effect on CORT release rates (Table 1).

Mass-corrected total bufadienolide quantity of tadpoles was significantly affected by the interaction between treatment length and exposure to exogenous chemicals (Table 1). In the 6-day treatments, tadpoles exposed to exoCORT contained significantly lower mcTBQ than control tadpoles and marginally non-significantly lower mcTBQ than tadpoles exposed to MTP, while the toxin content of tadpoles in the control group and those exposed to MTP did not differ significantly from each other (Table 2, Fig. 3B). Conversely, in tadpoles treated for 2 days, mcTBQ did not differ among treatments. Developmental stage of tadpoles significantly influenced mcTBQ, where more developed tadpoles contained lower amounts of bufadienolides per unit mass (Table 1, Fig. S3B). Predator treatment and its interactions had no significant effect on mcTBQ (Table 1).

Mass-corrected bufadienolide quantity significantly, positively correlated with CORT release rate ($r = 0.226$, $P < 0.001$, Fig. S4). However, this relationship was driven by developmental stage (Fig. S3): when we controlled for this covariate the correlation was no longer significant ($r = 0.049$, $P = 0.414$).

Discussion

Treating tadpoles with exogenous CORT resulted in significantly increased CORT release rates, regardless of treatment length, and this effect was accompanied by a decrease in bufadienolide toxin content after 6 days. Based on these results we can reject the hypothesis that the chemical defence of toad tadpoles is upregulated by increased CORT levels (H1, Fig. 1). The observed relationship between CORT release rate and bufadienolide content in our study is in concordance with previous findings indicating that exposure to the amphibian

chytrid fungus (*Batrachochytrium dendrobatidis*, Bd), an important amphibian pathogen, induces increased CORT levels in multiple amphibian species (e.g. Gabor et al., 2013b; Gabor et al., 2015; Gabor et al., 2018, but see Hammond et al., 2020), but decreases bufadienolide content of common toad tadpoles (Kásler et al., 2022; Ujszegi et al., 2021). We caution, however, against interpreting our result as CORT inhibiting bufadienolide synthesis, for the following reasons. First, multiple studies report that various environmental stressors (e.g. intraspecific competitors, predators, anthropogenic pollutants) known to increase CORT levels in animals (e.g. Berger et al., 2007; Braasch et al., 2014; Eraud et al., 2008; Gabor et al., 2018; Glennemeier and Denver, 2002a; Middlemis Maher et al., 2013; Monclús et al., 2018; Ye et al., 2015) also lead to increased bufadienolide production in toads (Bókony et al., 2017; Bókony et al., 2018; Hettyey et al., 2019; Üveges et al., 2021). Second, we found that individual variation in bufadienolide content was not correlated with variation in CORT release rate after controlling for developmental stage. We propose instead that our results on the effects of exoCORT treatment indirectly support the second hypothesis of our study (H2, Fig. 1), i.e. that bufadienolide synthesis is induced by upstream hormones of the HPI axis, such as ACTH (Dostanic-Larson et al., 2005; Fedorova et al., 1998). Although we did not measure ACTH in our experimental tadpoles, treating them with exoCORT most probably led to the suppression of ACTH discharge due to negative feedback (Gjerstad et al., 2018; Godoy et al., 2018; Herman et al., 2020), which may have ultimately resulted in decreased bufadienolide content. While our study cannot provide direct evidence for the role of upstream brain hormones in regulating bufadienolide production in toads (H2, Fig. 1), this remains a probable pathway, since previous studies suggested ACTH-induced synthesis of endogenous cardiotonic steroids (Dostanic-Larson et al., 2005; Laredo et al., 1994; Laredo et al., 1995; Li et al., 1994; Sophocleous et al., 2003; Vinge et al., 1993; Yamada et al., 1997), including unidentified bufadienolide-like compounds (Fedorova et al., 1998), in mammals.

We expected that treatment with MTP would lead to decreased CORT release rates compared to control animals, as in previous studies on amphibians (Glennemeier and Denver, 2002a; Glennemeier and Denver, 2002b). However, we found instead that MTP slightly, albeit significantly, increased CORT release rates compared to control tadpoles, regardless of treatment length. Such inconsistent effects of MTP treatment have been reported previously (Gabor et al., 2018; Glennemeier and Denver, 2002a; Kennedy et al., 2020; Kulkarni et al., 2017). There may be multiple, mutually non-exclusive explanations for the inability of MTP to reduce CORT. First, it is likely that the MTP concentration used in our study did not entirely impede CORT synthesis (Glennemeier and Denver, 2002a; Glennemeier and Denver, 2002b). Therefore, the elevated level of CORT observed in tadpoles treated with MTP may have simply been a consequence of compensatory ACTH release due to decreased CORT synthesis (Coppage et al., 1959; McEwen, 2007). Second, MTP inhibits aldosterone (ALDO) synthesis by inhibiting the transformation of 11-deoxycorticosterone to CORT and then that of CORT to ALDO (Gower, 1974). ALDO is a potent mineralocorticoid, regulating natriuresis and water retention (Liddle, 1958), and decreased levels of this hormone lead, among others, to increased urine volume (Brismar et al., 1985; Costello-Boerrigter et al., 2003; Liddle, 1958). Since in our study we measured CORT release rate of tadpoles, it is possible that the apparently increased CORT in response to MTP is a consequence of increased urine (and this way CORT) excretion due to the inhibition of ALDO synthesis.

We observed a decrease in bufadienolide content only after a 6-day exposure period to exoCORT. It is well known that systemic effects of GCs fundamentally differ when high levels are sustained only acutely *versus* chronically (Denver, 2009; Godoy et al., 2018; McEwen, 1998; McEwen, 2004; McEwen and Wingfield, 2003; Middlemis Maher et al., 2013; Sterling and Eyer, 1988). Effects of acute exoCORT exposure, or spikes in innate CORT levels, are relatively quickly counteracted by other physiological processes, and the

concentrations of CORT and upstream hormones return to baseline levels (McEwen, 1998; McEwen et al., 2015; McEwen and Wingfield, 2003; Romero et al., 2009). On the other hand, chronically high levels of CORT can induce emergency countermeasures or even pathological changes in a multitude of physiological processes (Denver, 2009; Godoy et al., 2018; McEwen, 1998; McEwen, 2004; McEwen and Wingfield, 2003; Sterling and Eyer, 1988). Such effects of the 6-day exoCORT treatment might have led to a decrease in bufadienolide content by e.g. the breakdown of the acidic bile-acid pathway of steroidogenesis (Fedorova et al., 2015), or depletion of toxin reserves due to catabolism of toxin compounds for energy release.

Lastly, exposure to predation cues did not result in increased CORT release rates in experimental tadpoles. It is possible that cues on the presence of predators are relatively weak triggers of the endocrine stress response in common toad tadpoles. For instance, induced responses against predators in e.g. behaviour and morphology are accompanied by increased CORT levels in amphibians (Kulkarni and Gramapurohit, 2017; Middlemis Maher et al., 2013), but common toad tadpoles display less intense behavioural and morphological plasticity to the presence of predators compared to larvae of other amphibians (Lardner, 2000; Laurila et al., 1998; Van Buskirk, 2002). This is possibly due to the effectiveness of bufadienolides providing defence against various predator species even at non-induced levels (Üveges et al., 2019). Future studies similar to the current experiment should use a higher concentration of predator cues or other types of (possibly more effective) stressors to trigger the upregulation of CORT release rates in toad tadpoles.

Bufadienolides are emerging as important compounds for multiple fields of research. They are considered to be important ion-transport regulators and agents of cell signalling (Lichtstein et al., 2012; Lingrel, 2010; Pierre and Xie, 2006; Schoner and Scheiner-Bobis, 2007), however, to our knowledge, studies investigating the housekeeping aspect of

bufadienolides are entirely lacking outside of mammals. Therefore, it would be worthwhile for future studies to also focus on non-model vertebrates. Investigating how bufadienolide production influences other physiological processes would also be welcome. For instance, bufadienolides can also inhibit steroidogenesis (Kau et al., 2012), therefore, changes in bufadienolide levels may also influence sex-hormone synthesis. The consequences of this interaction are currently not known, but could potentially impact individual fitness and thus population dynamics and survival in the long run (Bókonyi et al., 2019). Deeper understanding about the regulation of these compounds might also benefit native populations suffering from invasive toad species (Licata et al., 2019; Shine, 2010), by e.g. potentially leading to methods for selective inhibition of bufadienolide synthesis, thereby facilitating survival of toad predators (Caller and Brown, 2013; Greenlees et al., 2010; Phillips and Shine, 2006). Last but not least, research about how these housekeeping molecules became effective chemical weapons may also provide important insights for molecular evolution. Clearly, more research is needed to shed light on the background of phenotypically plastic bufadienolide synthesis, and its potential effects on the physiology of animals, as well as its ecological and evolutionary consequences.

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Data availability: Dataset of the study is available on the figshare digital repository at <https://doi.org/10.6084/m9.figshare.15090339> (Üveges et al., 2023).

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References

- Achtymichuk, G. H., Crane, A. L., Simko, O. M., Stevens, H. E. F. and Ferrari, M. C. O. (2022). The choice of euthanasia techniques can affect experimental results in aquatic behavioural studies. *Animal Behaviour* **185**, 1-8.
- Arbuckle, K., Brockhurst, M. and Speed, M. P. (2013). Does chemical defence increase niche space? A phylogenetic comparative analysis of the Musteloidea. *Evolutionary Ecology* **27**, 863-881.
- Barnhart, K., Forman, M. E., Umile, T. P., Kueneman, J., McKenzie, V., Salinas, I., Minbiole, K. P. C. and Woodhams, D. C. (2017). Identification of bufadienolides from the boreal toad, *Anaxyrus boreas*, active against a fungal pathogen. *Microbial Ecology* **74**, 990-1000.
- Bates, D., Mächler, M., Bolker, B. M. and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**, 1-48.
- Baugh, A. T., Bastien, B., Still, M. B. and Stowell, N. (2018). Validation of water-borne steroid hormones in a tropical frog (*Physalaemus pustulosus*). *General and Comparative Endocrinology* **261**, 67-80.
- Benard, M. F. and Fordyce, J. A. (2003). Are induced defenses costly? Consequences of predator-induced defenses in western toads, *Bufo boreas*. *Ecology* **84**, 68-78.
- Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B: Statistical Methods* **57**, 289-300.
- Berger, S., Wikelski, M., Romero, L. M., Kalko, E. K. V. and Rödl, T. (2007). Behavioral and physiological adjustments to new predators in an endemic island species, the Galápagos marine iguana. *Hormones and Behavior* **52**, 653-663.
- Blum, M. S. (1981). Chemical defenses of Arthropods. New York, USA: Academic Press Inc. .
- Bókony, V., Mikó, Z., Mórícz, Á. M., Krüzselyi, D. and Hettyey, A. (2017). Chronic exposure to a glyphosate-based herbicide makes toad larvae more toxic. *Proceedings of the Royal Society B: Biological Sciences* **284**, 20170493.

Bókony, V., Üveges, B., Móricz, Á. M. and Hettyey, A. (2018). Competition induces increased toxin production in toad larvae without allelopathic effects on heterospecific tadpoles. *Functional Ecology* **32**, 667-675.

Bókony, V., Üveges, B., Verebélyi, V., Ujhegyi, N. and Móricz, Á. M. (2019). Toads phenotypically adjust their chemical defences to anthropogenic habitat change. *Scientific Reports* **9**, 3163.

Braasch, A., Becker, P. H. and Groothuis, T. G. G. (2014). Response of testosterone and corticosterone plasma levels to the challenge of sibling competition: A study in common terns. *General and Comparative Endocrinology* **204**, 95-103.

Brismar, K., Werner, S., Thorén, M. and Wetterberg, L. (1985). Metyrapone: an agent for melatonin as well as ACTH and cortisol secretion. *Journal of Endocrinological Investigation* **8**, 91-95.

Brodie, E. D., III. (2009). Toxins and venoms. *Current Biology* **19**, R931-R935.

Brodie, E. D., III and Brodie, E. D., Jr. (1990). Tetrodotoxin resistance in garter snakes: An evolutionary response of predators to dangerous prey. *Evolution* **44**, 651-659.

Caller, G. and Brown, C. (2013). Evolutionary responses to invasion: Cane toad sympatric fish show enhanced avoidance learning. *PLoS ONE* **8**, e54909-e54909.

Casewell, N. R., Wüster, W., Vonk, F. J., Harrison, R. A. and Fry, B. G. (2013). Complex cocktails: the evolutionary novelty of venoms. *Trends in Ecology & Evolution* **28**, 219-29.

Chen, L. and Huang, G.-z. (2013). Poisoning by toxic animals in China - 18 autopsy case studies and a comprehensive literature review. *Forensic Science International* **232**, e12-e23.

Coppage, W. S., Jr., Island, D., Smith, M. and Liddle, G. W. (1959). Inhibition of aldosterone secretion and modification of electrolyte excretion in man by a chemical inhibitor of 11 β -hydroxylation. *Journal of Clinical Investigation* **38**, 2101-2110.

Costello-Boerriqter, L. C., Boerriqter, G. and Burnett, J. C. (2003). Revisiting salt and water retention: New diuretics, aquaretics, and natriuretics. *Medical Clinics of North America* **87**, 475-491.

Cunha Filho, G. A., Schwartz, C. A., Resck, I. S., Murta, M. M., Lemos, S. S., Castro, M. S., Kyaw, C., Pires, O. R., Jr., Leite, J. R., Bloch, C., Jr. 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**, 777-82.

Delfino, G., Brizzi, R. and Feri, L. (1995). Chemical skin defence in *Bufo bufo*: an ultrastructural study during ontogenesis. *Zoologischer Anzeiger* **234**, 101-111.

Denver, R. J. (2009). Structural and functional evolution of vertebrate neuroendocrine stress systems. *Annals of the New York Academy of Sciences* **1163**, 1-16.

Dostanic-Larson, I., Van Huisse, J. W., Lorenz, J. N. and Lingrel, J. B. (2005). The highly conserved cardiac glycoside binding site of Na,K-ATPase plays a role in blood pressure regulation. *Proceedings of the National Academy of Sciences* **102**, 15845-15850.

Eraud, C., Trouvé, C., Danó, S., Chastel, O. and Faivre, B. (2008). Competition for resources modulates cell-mediated immunity and stress hormone level in nestling collared doves (*Streptopelia decaocto*). *General and Comparative Endocrinology* **155**, 542-551.

Fedorova, O. V., Anderson, D. E. and Bagrov, A. Y. (1998). Plasma marinobufagenin-like and ouabain-like immunoreactivity in adrenocorticotropin-treated rats. *American Journal of Hypertension* **11**, 796-802.

Fedorova, O. V., Zernetkina, V. I., Shilova, V. Y., Grigorova, Y. N., Juhasz, O., Wei, W., Marshall, C. A., Lakatta, E. G. and Bagrov, A. Y. (2015). Synthesis of an endogenous steroidal Na pump inhibitor marinobufagenin, implicated in human cardiovascular diseases, is initiated by CYP27A1 via bile acid pathway. *Circulation: Cardiovascular Genetics* **8**, 736-745.

Forsburg, Z. R., Goff, C. B., Perkins, H. R., Robicheaux, J. A., Almond, G. F. and Gabor, C. R. (2019). Validation of water-borne cortisol and corticosterone in tadpoles: Recovery rate from an acute stressor, repeatability, and evaluating rearing methods. *General and Comparative Endocrinology* **281**, 145-152.

Fox, J. and Weisberg, S. (2019). An R companion to applied regression. Thousand Oaks CA: Sage.

- Gabor, C. R., Bosch, J., Fries, J. N. and Davis, D. R.** (2013a). A non-invasive water-borne hormone assay for amphibians. *Amphibia-Reptilia* **34**, 151-162.
- Gabor, C. R., Fisher, M. C. and Bosch, J.** (2013b). A non-invasive stress assay shows that tadpole populations infected with *Batrachochytrium dendrobatidis* have elevated corticosterone levels. *PLoS ONE* **8**, e56054.
- Gabor, C. R., Fisher, M. C. and Bosch, J.** (2015). Elevated corticosterone levels and changes in amphibian behavior are associated with *Batrachochytrium dendrobatidis* (Bd) infection and Bd lineage. *PLoS ONE* **10**, e0122685.
- Gabor, C. R., Knutie, S. A., Roznik, E. A. and Rohr, J. R.** (2018). Are the adverse effects of stressors on amphibians mediated by their effects on stress hormones? *Oecologia* **186**, 393-404.
- Gabor, C. R., Perkins, H. R., Heitmann, A. T., Forsburg, Z. R. and Aspbury, A. S.** (2019). Roundup™ with corticosterone functions as an infodisruptor to antipredator response in tadpoles. *Frontiers in Ecology and Evolution* **7**, 114.
- Gabor, C. R., Zabierek, K. C., Kim, D. S., da Barbiano, L. A., Mondelli, M. J., Bendik, N. F. and Davis, D. R.** (2016). A non-invasive water-borne assay of stress hormones in aquatic salamanders. *Copeia* **104**, 172-181.
- Gjerstad, J. K., Lightman, S. L. and Spiga, F.** (2018). Role of glucocorticoid negative feedback in the regulation of HPA axis pulsatility. *Stress* **21**, 403-416.
- Glennemeier, K. A. and Denver, R. J.** (2002a). Role for corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition. *Journal of Experimental Zoology* **292**, 32-40.
- Glennemeier, K. A. and Denver, R. J.** (2002b). Small changes in whole-body corticosterone content affect larval *Rana pipiens* fitness components. *General and Comparative Endocrinology* **127**, 16-25.
- Godoy, L. D., Rossignoli, M. T., Delfino-Pereira, P., Garcia-Cairasco, N. and de Lima Umeoka, E. H.** (2018). A Comprehensive overview on stress neurobiology: Basic concepts and clinical implications. *Frontiers in Behavioral Neuroscience* **12**, 1-23.
- Gosner, K. L.** (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**, 183-190.
- Gower, D. B.** (1974). Modifiers of steroid-hormone metabolism: A review of their chemistry, biochemistry and clinical applications. *Journal of Steroid Biochemistry* **5**, 501-523.
- Greenlees, M. J., Phillips, B. L. and Shine, R.** (2010). Adjusting to a toxic invader: Native Australian frogs learn not to prey on cane toads. *Behavioral Ecology* **21**, 966-971.
- Hagman, M., Hayes, R. A., Capon, R. J. and Shine, R.** (2009). Alarm cues experienced by cane toad tadpoles affect post-metamorphic morphology and chemical defences. *Functional Ecology* **23**, 126-132.
- Hammond, T. T., Blackwood, P. E., Shablin, S. A. and Richards-Zawacki, C. L.** (2020). Relationships between glucocorticoids and infection with *Batrachochytrium dendrobatidis* in three amphibian species. *General and Comparative Endocrinology* **285**, 113269.
- Hayes, R. A., Crossland, M. R., Hagman, M., Capon, R. J. and Shine, R.** (2009). Ontogenetic variation in the chemical defenses of cane toads (*Bufo marinus*): toxin profiles and effects on predators. *Journal of Chemical Ecology* **35**, 391-9.
- Hayes, T. B. and Wu, T. H.** (1995). Interdependence of corticosterone and thyroid hormones in toad larvae (*Bufo boreas*). II. Regulation of corticosterone and thyroid hormones. *Journal of Experimental Zoology* **271**, 103-111.
- Herman, J. P., Nawreen, N., Smail, M. A. and Cotella, E. M.** (2020). Brain mechanisms of HPA axis regulation: Neurocircuitry and feedback in context Richard Kvetnansky lecture. *Stress* **23**, 617-632.
- Hettyey, A., Tóth, Z., Thonhauser, K. E., Frommen, J. G., Penn, D. J. and 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.

- Hettyey, A., Üveges, B., Móricz, Á. M., Drahos, L., Capon, R. J., Van Buskirk, J., Tóth, Z. and Bókony, V. (2019). Predator-induced changes in the chemical defence of a vertebrate. *Journal of Animal Ecology* **00**, 1-12.
- Hossie, T. J., Hassall, C., Knee, W. and Sherratt, T. N. (2013). Species with a chemical defence, but not chemical offence, live longer. *Journal of Evolutionary Biology* **26**, 1598-1602.
- Kamboj, A., Rathour, A. and Mandeep, K. (2013). Bufadienolides and their medicinal utility: A review. *International Journal of Pharmacy and Pharmaceutical Sciences* **5**, 20-27.
- Kásler, A., Ujszegi, J., Holly, D., Üveges, B., Móricz, Á. M., Herczeg, D. and Hettyey, A. (2022). Metamorphic common toads keep chytrid infection under control, but at a cost. *Journal of Zoology* **317**, 159-169.
- Kau, M.-M., Wang, J.-R., Tsai, S.-C., Yu, C.-H. and Wang, P. S. (2012). Inhibitory effect of bufalin and cinobufagin on steroidogenesis via the activation of ERK in human adrenocortical cells. *British Journal of Pharmacology* **165**, 1868-1876.
- Kennedy, C. L. M., Carter, S. D., Mifsud, K. R. and Reul, J. M. H. M. (2020). Unexpected effects of metyrapone on corticosteroid receptor interaction with the genome and subsequent gene transcription in the hippocampus of male rats. *Journal of Neuroendocrinology* **32**, e12820.
- Kim, S. (2015). ppcor: An R package for a fast calculation to semi-partial correlation coefficients. *Communications for Statistical Applications and Methods* **22**, 665-674.
- Koolhaas, J. M., Bartolomucci, A., Buwalda, B., de Boer, S. F., Flügge, G., Korte, S. M., Meerlo, P., Murison, R., Olivier, B., Palanza, P. et al. (2011). Stress revisited: A critical evaluation of the stress concept. *Neuroscience & Biobehavioral Reviews* **35**, 1291-1301.
- Kulkarni, P. S. and Gramapurohit, N. P. (2017). Effect of corticosterone on larval growth, antipredator behaviour and metamorphosis of *Hylarana indica*. *General and Comparative Endocrinology* **251**, 21-29.
- Kulkarni, S. S., Denver, R. J., Gomez-Mestre, I. and Buchholz, D. R. (2017). Genetic accommodation via modified endocrine signalling explains phenotypic divergence among spadefoot toad species. *Nature Communications* **8**, 993.
- Lardner, B. (2000). Morphological and life history responses to predators in larvae of seven anurans. *Oikos* **88**, 169-180.
- Laredo, J., Hamilton, B. P. and Hamlyn, J. M. (1994). Ouabain is secreted by bovine adrenocortical cells. *Endocrinology* **135**, 794-797.
- Laredo, J., Hamilton, B. P. and Hamlyn, J. M. (1995). Secretion of endogenous ouabain from bovine adrenocortical cells: Role of the zona glomerulosa and zona fasciculata. *Biochemical and Biophysical Research Communications* **212**, 487-493.
- Laurila, A., Kujasalo, J. and Ranta, E. (1997). Different antipredator behaviour in two anuran tadpoles: effects of predator diet. *Behavioral Ecology and Sociobiology* **40**, 329-336.
- Laurila, A., Kujasalo, J. and Ranta, E. (1998). Predator-induced changes in life history in two anuran tadpoles: effects of predator diet. *Oikos* **83**, 307-317.
- Lenth, R. V., Singmann, H., Love, J., Buerkner, P. and Herve, M. (2019). emmeans: Estimated marginal means, aka least-squares means. CRAN.
- Li, M., Wong, K.-S., Martin, A. and Whitworth, J. A. (1994). Adrenocorticotrophin-induced hypertension in rats: Role of progesterone and digoxin-like substances. *American Journal of Hypertension* **7**, 59-68.
- Licata, F., Ficetola, G. F., Freeman, K., Mahasoa, R. H., Ravololonarivo, V., Solofo Niaina Fidy, J. F., Koto-Jean, A. B., Nahavitsara, E. R., Andreone, F. and Crottini, A. (2019). Abundance, distribution and spread of the invasive Asian toad *Duttaphrynus melanostictus* in eastern Madagascar. *Biological Invasions* **21**, 1615-1626.
- Lichtstein, D., Rosen, H. and Dvela, M. (2012). Cardenolides and bufadienolides as hormones: What is missing? *American Journal of Physiology - Renal Physiology* **302**, F957-F958.
- Liddle, G. W. (1958). Aldosterone antagonists. *A.M.A. Archives of Internal Medicine* **102**, 998-1004.

- Lingrel, J. B.** (2010). The physiological significance of the cardiotoxic steroid/ouabain-binding site of the Na,K-ATPase. *Annual Review of Physiology* **72**, 395-412.
- Llewellyn, J., Bell, K., Schwarzkopf, L., Alford, R. A. and Shine, R.** (2012). Ontogenetic shifts in a prey's chemical defences influence feeding responses of a snake predator. *Oecologia* **169**, 965-973.
- McEwen, B. S.** (1998). Stress, adaptation, and disease: Allostasis and allostatic load. *Annals of the New York Academy of Sciences* **840**, 33-44.
- McEwen, B. S.** (2004). Protection and damage from acute and chronic stress: Allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders. *Annals of the New York Academy of Sciences* **1032**, 1-7.
- McEwen, B. S.** (2007). Physiology and neurobiology of stress and adaptation: Central role of the brain. *Physiological Reviews* **87**, 873-904.
- McEwen, B. S., Gray, J. D. and Nasca, C.** (2015). Redefining neuroendocrinology: Stress, sex and cognitive and emotional regulation. *Journal of Endocrinology* **226**, T67-T83.
- McEwen, B. S. and Wingfield, J. C.** (2003). The concept of allostasis in biology and biomedicine. *Hormones and Behavior* **43**, 2-15.
- Middlemis Maher, J., Werner, E. E. and Denver, R. J.** (2013). Stress hormones mediate predator-induced phenotypic plasticity in amphibian tadpoles. *Proceedings of the Royal Society B: Biological Sciences* **280**, 20123075.
- Monclús, L., Ballesteros-Cano, R., De La Puente, J., Lacorte, S. and Lopez-Bejar, M.** (2018). Influence of persistent organic pollutants on the endocrine stress response in free-living and captive red kites (*Milvus milvus*). *Environmental Pollution* **242**, 329-337.
- Nelson, D. W. M., Crossland, M. R. and Shine, R.** (2010). Indirect ecological impacts of an invasive toad on predator-prey interactions among native species. *Biological Invasions* **12**, 3363-3369.
- Nelson, D. W. M., Crossland, M. R. and Shine, R.** (2011). Foraging responses of predators to novel toxic prey: Effects of predator learning and relative prey abundance. *Oikos* **120**, 152-158.
- Pawlik, J. R.** (1993). Marine invertebrate chemical defenses. *Chemical Reviews* **93**, 1911-1922.
- Payne, A. H. and Hales, D. B.** (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocrine Reviews* **25**, 947-970.
- Payton, M. E., Greenstone, M. H. and Schenker, N.** (2003). Overlapping confidence intervals or standard error intervals: What do they mean in terms of statistical significance? *Journal of Insect Science* **3**, 34.
- Phillips, B. L. and Shine, R.** (2006). An invasive species induces rapid adaptive change in a native predator: Cane toads and black snakes in Australia. *Proceedings of the Royal Society B: Biological Sciences* **273**, 1545-1550.
- Pierre, S. V. and Xie, Z.** (2006). The Na,K-ATPase receptor complex. *Cell Biochemistry and Biophysics* **46**, 303-315.
- Pike, N.** (2011). Using false discovery rates for multiple comparisons in ecology and evolution. *Methods in Ecology and Evolution* **2**, 278-282.
- Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. and R Core Team.** (2017). nlme: Linear and nonlinear mixed effects models. Vienna, Austria: The R Project for Statistical Computing.
- R Development Core Team.** (2017). A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.
- Rohr, J. R., Raffel, T. R., Halstead, N. T., McMahon, T. A., Johnson, S. A., Boughton, R. K. and Martin, L. B.** (2013). Early-life exposure to a herbicide has enduring effects on pathogen-induced mortality. *Proceedings of the Royal Society B: Biological Sciences* **280**, 20131502.
- Romero, L. M., Dickens, M. J. and Cyr, N. E.** (2009). The reactive scope model — A new model integrating homeostasis, allostasis, and stress. *Hormones and Behavior* **55**, 375-389.
- Schoeppner, N. M. and Relyea, R. A.** (2005). Damage, digestion, and defence: the roles of alarm cues and kairomones for inducing prey defences. *Ecology Letters* **8**, 505-512.

- Schoner, W. and Scheiner-Bobis, G.** (2007). Endogenous and exogenous cardiac glycosides and their mechanisms of action. *American Journal of Cardiovascular Drugs* **7**, 173-189.
- Shine, R.** (2010). The ecological impact of invasive cane toads (*Bufo marinus*) in Australia. *Quarterly Review of Biology* **85**, 253-291.
- Sophocleous, A., Elmatzoglou, I. and Souvatzoglou, A.** (2003). Circulating endogenous digitalis-like factor(s) (EDLF) in man is derived from the adrenals and its secretion is ACTH-dependent. *Journal of Endocrinological Investigation* **26**, 668-674.
- Sterling, P. and Eyer, J.** (1988). Allostasis: A new paradigm to explain arousal pathology. In *Handbook of life stress, cognition and health*, eds. S. Fisher and J. Reason), pp. 629-649: John Wiley & Sons.
- Tempone, A. G., Pimenta, D. C., Lebrun, I., Sartorelli, P., Taniwaki, N. N., de Andrade Jr, H. F., Antoniazzi, M. M. and Jared, C.** (2008). Antileishmanial and antitrypanosomal activity of bufadienolides isolated from the toad *Rhinella jimi* parotoid macrogland secretion. *Toxicon* **52**, 13-21.
- Toledo, R. C. and Jared, C.** (1995). Cutaneous granular glands and amphibian venoms. *Comparative Biochemistry and Physiology A: Physiology* **111**, 1-29.
- Ujszegi, J., Ludányi, K., Móricz, Á. M., Krüzselyi, D., Drahos, L., Drexler, T., Németh, M. Z., Vörös, J., Garner, T. W. J. and Hettyey, A.** (2021). Exposure to *Batrachochytrium dendrobatidis* affects chemical defences in two anuran amphibians, *Rana dalmatina* and *Bufo bufo*. *BMC Ecology and Evolution* **21**, 135.
- Ujszegi, J., Móricz, Á. M., Krüzselyi, D. and Hettyey, A.** (2017). Skin toxin production of toads changes during early ontogeny but is not adjusted to the microbiota of the aquatic environment. *Evolutionary Ecology* **31**, 925-936.
- Ujvari, B., Casewell, N. R., Sunagar, K., Arbuckle, K., Wüster, W., Lo, N., O'Meally, D., Beckmann, C., King, G. F., Deplazes, E. et al.** (2015). Widespread convergence in toxin resistance by predictable molecular evolution. *Proceedings of the National Academy of Sciences* **112**, 11911-11916.
- Üveges, B., Basson, A. C., Móricz, Á. M., Bókony, V. and Hettyey, A.** (2021). Chemical defence effective against multiple enemies: Does the response to conspecifics alleviate the response to predators? *Functional Ecology* **35**, 2294-2304.
- Üveges, B., Fera, G., Móricz, Á. M., Krüzselyi, D., Bókony, V. and Hettyey, A.** (2017). Age- and environment-dependent changes in chemical defences of larval and post-metamorphic toads. *BMC Evolutionary Biology* **17**, 137.
- Üveges, B., Kalina, C., Szabó, K., Móricz, Á. M., Holly, D., Gabor, C. R., Hettyey, A. and Bókony, V.** (2023). Data: Does stress make toads more toxic? An experimental study on the hormonal regulation of bufadienolide toxin synthesis. figshare.
- Üveges, B., Szederkényi, M., Mahr, K., Móricz, Á. M., Krüzselyi, D., Bókony, V., Hoi, H. and Hettyey, A.** (2019). Chemical defense of toad tadpoles under risk by four predator species. *Ecology and Evolution* **9**, 6287-6299.
- Van Buskirk, J.** (2002). A comparative test of the adaptive plasticity hypothesis: relationships between habitat and phenotype in anuran larvae. *The American Naturalist* **160**, 87-102.
- Verebélyi, V.** (2017). Barna varangy (*Bufo bufo*) szaporodási sikerének és szennyezőanyag rezisztenciájának összehasonlítása különböző szennyezett élőhelytípusok között. In *Institute of Biology, Department of Ecology*. Budapest, Hungary: University of Veterinary Medicine Budapest.
- Vinge, E., Erfurth, E.-M. and Odar-Cederlöf, I.** (1993). Immunoreactive endogenous digoxin-like substances: Plasma levels are dependent on the hypothalamic-pituitary-adrenal axis for release and on kidney function for elimination. *Journal of Cardiovascular Pharmacology* **22** (Suppl. 2), S112-3.
- von Frisch, K.** (1942). Über einen Schreckstoff der Fischhaut und seine biologische Bedeutung. *Zeitschrift für Vergleichende Physiologie* **29**, 46-145.
- Webb, J. K., Brown, G. P., Child, T., Greenlees, M. J., Phillips, B. I. and Shine, R.** (2008). A native dasyurid predator (common planigale, *Planigale maculata*) rapidly learns to avoid a toxic invader. *Austral Ecology* **33**, 821-829.

Yamada, K., Goto, A. and Omata, M. (1997). Adrenocorticotropin-induced hypertension in rats: Role of ouabain-like compound. *American Journal of Hypertension* **10**, 403-408.

Ye, L., Hu, Z., Wang, H., Zhu, H., Dong, Z., Jiang, W., Zhao, H., Li, N., Mi, W., Wang, W. et al. (2015). Tris-(2,3-dibromopropyl) isocyanurate, a new emerging pollutant, impairs cognition and provokes depression-like behaviors in adult rats. *PLoS ONE* **10**, e0140281.

Young, S. D., Gavel, M. J., Gutierrez-Villagomez, J. M., Forbes, M. R. and Robinson, S. A. (2020). Assessment of sublethal ecotoxicity of solvents on larvae of a model native amphibian (*Lithobates pipiens*). *Journal of Applied Toxicology* **40**, 483-492.

ORIGINAL UNEDITED MANUSCRIPT

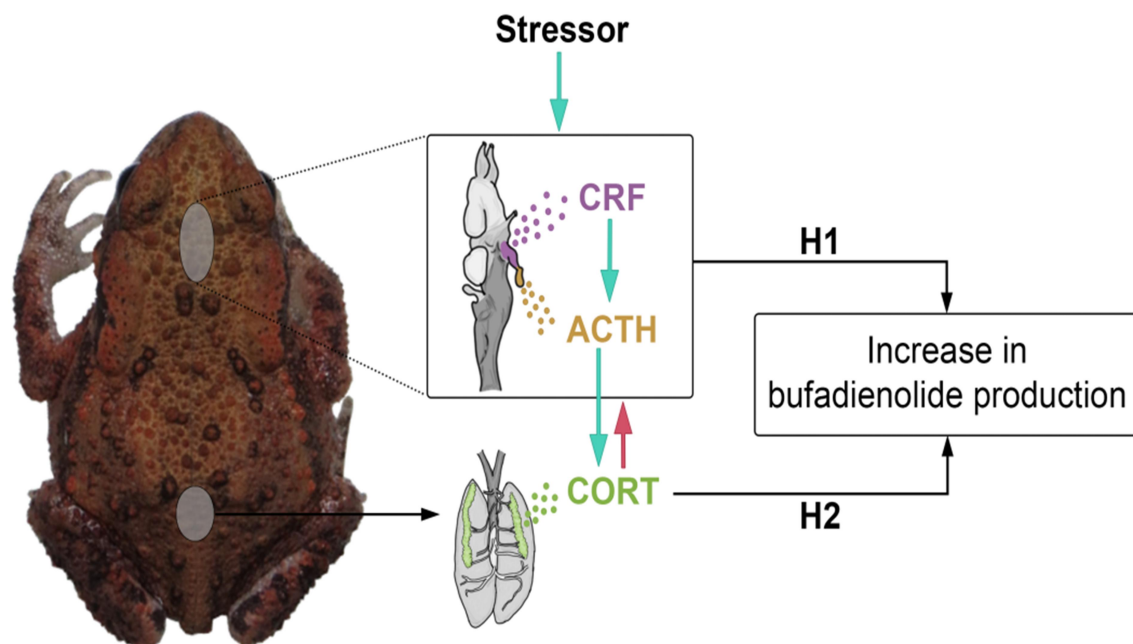


Fig. 1: Schematic representation of the HPI axis and potential physiological regulatory pathways of bufadienolide synthesis in common toads. Green arrows indicate upregulation, whereas a red arrow indicates negative feedback. Abbreviations: CRF: corticotropin-releasing factor, ACTH: adrenocorticotrophic hormone, CORT: corticosterone, H1: hypothesis 1, H2: hypothesis 2. Photo of juvenile toad by Nikolett Ujhegyi, used with permission.

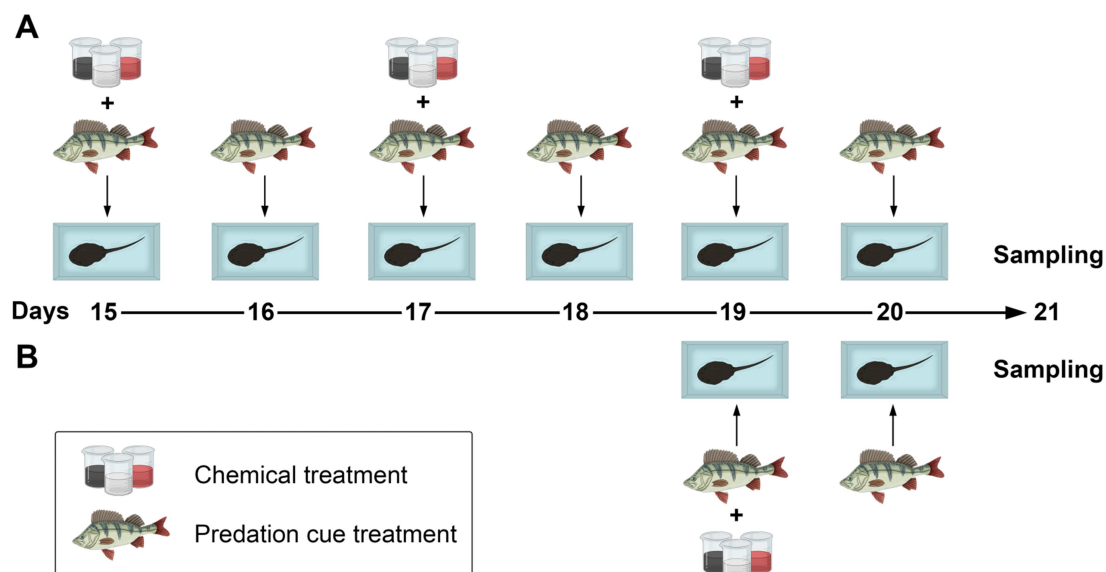


Fig. 2: Experimental design applied to containers of tadpoles during the last week of the experiment (treatment period), beginning with day 15. **A)** treatment length 6 days, **B)** treatment length 2 days. Beakers represent changes of the tadpoles' rearing water with water containing exogenous chemicals (CORT, MTP) or the control solution. Perch represent addition of reconstituted soft-water (control tadpoles) or water from the fish's tank + tadpole homogenate. Icons of beakers used in the figure are licensed by Biorender.

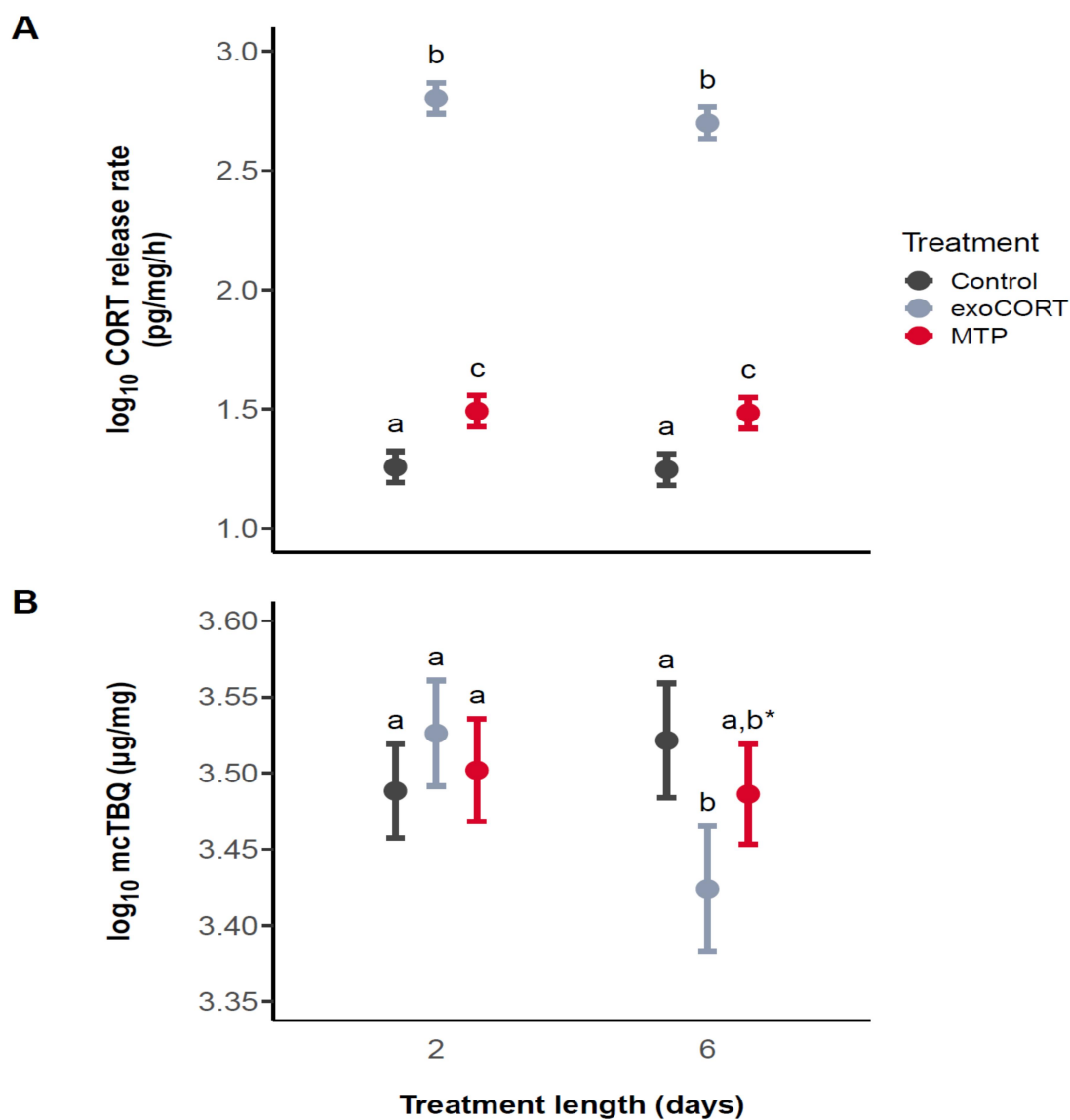


Fig. 3: Effects of treatments (exoCORT: exogenous corticosterone, MTP: metyrapone) on corticosterone (CORT) release rate (**A**) and mass-corrected total bufadienolide content (mcTBQ, **B**) of toad tadpoles. Error bars represent estimated means and 84% confidence intervals (CI) calculated from linear mixed-effects models ($N = 283$). Non-overlapping CIs indicate significant differences between groups after correction for false discovery rate. Letters above error bars represent pairwise comparisons; groups not sharing any letter differ significantly ($P < 0.05$). An asterisk depicts a marginally non-significant difference ($P = 0.071$) between groups treated with exoCORT and MTP.

Table 1: Treatment effects (type-2 analysis of deviance) on corticosterone (CORT) release rate and mass-corrected bufadienolide quantity (mcTBQ) of toad tadpoles. Significant effects are highlighted in bold.

Response	Effect	χ^2	df	P
CORT release rate	developmental stage	57.776	1	<0.001
	exogenous chemicals	2674.305	2	<0.001
	predation cues	0.456	1	0.499
	treatment length	2.365	1	0.124
	exogenous chemicals \times predation cues	3.024	2	0.221
	exogenous chemicals \times treatment length	3.079	2	0.215
	predation cues \times treatment length	0.193	1	0.661
	exogenous chemicals \times predation cues \times treatment length	0.91	2	0.634
mcTBQ	developmental stage	272.422	1	<0.001
	exogenous chemicals	0.559	2	0.756
	predation cues	0.014	1	0.906
	treatment length	1.257	1	0.262
	exogenous chemicals \times predation cues	0.071	2	0.965
	exogenous chemicals \times treatment length	10.327	2	0.006
	predation cues \times treatment length	2.28	1	0.131
	exogenous chemicals \times predation cues \times treatment length	3.44	2	0.179

Table 2: Effects of exogenous chemicals on corticosterone (CORT) release rate and mass-corrected total bufadienolide quantity (mcTBQ) of toad tadpoles. Estimates of linear contrasts are differences between the mean values of tadpoles exposed to the solvent control (EtOH), exogenous corticosterone (exoCORT) or metyrapone (MTP). *P* values were corrected using the FDR method for each dependent variable. Significant differences are highlighted in bold.

Response	Treatment length (days)	contrast	estimate	SE	df	<i>t</i>	<i>P</i>
CORT release rate	2	exoCORT – MTP	-1.312	0.044	261	-30.067	<0.001
	2	EtOH – exoCORT	1.546	0.044	261	35.423	<0.001
	2	EtOH – MTP	0.234	0.044	261	5.376	<0.001
	6	exoCORT – MTP	-1.215	0.044	262	-27.711	<0.001
	6	EtOH – exoCORT	1.453	0.045	263	32.653	<0.001
	6	EtOH – MTP	0.238	0.043	262	5.491	<0.001
mcTBQ	2	exoCORT – MTP	-0.024	0.028	264	-0.855	0.590
	2	EtOH – exoCORT	0.038	0.027	264	1.401	0.487
	2	EtOH – MTP	0.014	0.026	264	0.518	0.605
	6	exoCORT – MTP	0.062	0.031	264	1.991	0.071
	6	EtOH – exoCORT	-0.097	0.034	264	-2.898	0.012
	6	EtOH – MTP	-0.035	0.030	264	-1.194	0.233