Title: Chemical defence effective against multiple enemies: does the response to conspecifics alleviate the response to predators?

Authors: Bálint Üveges[1*], Anna C. Basson[1,2], Ágnes M. Móricz[3], Veronika Bókony[1], Attila Hettyey[1]

[*]correspondence: uveges.balint@yahoo.de, Plant Protection Institute, Centre for Agricultural Research, 1022, Budapest, Herman Ottó út. 15.

Affiliations: [1]Lendület Evolutionary Ecology Research Group, Plant Protection Institute, Centre for Agricultural Research, 1022, Herman Ottó út 15, Budapest, Hungary
[2]Department of Systematic Zoology and Ecology, Eötvös Loránd University, 1117 Budapest, Pázmány Péter sétány 1/A, Budapest, Hungary
[3]Department of Pathophysiology, Plant Protection Institute, Centre for Agricultural Research, 1022, Herman Ottó út 15, Budapest, Hungary

Conflict of Interest: The authors declare that they have no conflicts of interest.

Authors' contributions: BÜ, VB and AH conceived and designed the study, BÜ and ACB conducted the experiment, collected data and prepared samples for chemical analysis which were run by ÁMM. BÜ, ACB and VB analysed data; BÜ, VB and AH wrote the manuscript, and all authors gave approval for publication.

Data availability: Dataset of the study available from the figshare digital repository at https://figshare.com/s/c43acbb01d493a7f10e5 (https://dx.doi.org/ 10.6084/m9.figshare.11353637; Üveges et al., 2021).

Abstract

1. Animals living in groups with high conspecific densities typically decrease their level of plastic anti-predatory defence, because its benefits diminish with reduced *per capita* predation risk (a benefit of aggregation), whereas its costs increase due to intensifying competition and increased infection risk. Furthermore, phenotypic responses that provide protection from predators are also often disadvantageous against competitors and infections.

2. Such a trade-off may be absent when the same phenotype provides an effective defence against both predators and competitors, as is the case with some chemical defences. For such multifunctional defensive traits, both predation risk and high conspecific density are expected to increase defence expression, while simultaneous exposure to both predators and conspecifics may result in non-additive effects whereby the defence level induced by two enemies is lower than the sum of responses induced by either of them alone.

3. We tested this theoretical prediction by studying the effects of multiple enemies on chemical defence in a vertebrate animal. We investigated patterns of change in toxin production of common toad *Bufo bufo* tadpoles following exposure to different conspecific densities and the simultaneous presence or absence of chemical cues on predation risk.

4. We found that tadpoles significantly increased their production of bufadienolide toxins in response to high tadpole density, as well as to predation risk when tadpole density was low. Although the response in bufadienolide production to predation risk was not significant at high tadpole density, the magnitude of anti-predatory response did not differ significantly between low and high tadpole densities.

5. These results show that toad tadpoles adjust their chemical defence to conspecific density and to predation risk simultaneously, and these two effects are more likely additive than non-additive, at least within the range of densities and predation-risk levels studied here. Nevertheless, the trend we found suggests that toxin levels induced by very high conspecific density might weaken the chemical response to predators, which is relevant for the evolutionary ecology of chemical defences, as well as for the conservation of fauna impacted by toxic invaders.

Keywords: antipredator response, anuran amphibian, chemical defence, defensive synergy, inducible defence, multiple stressors, phenotypic plasticity, toxins

Introduction

Predation is one of the most important selective forces driving evolutionary change, therefore, organisms must adapt their defences to the actual levels of predation pressure to maximise their fitness. This adaptation is possible in part due to prey's ability to respond to environmental variability by phenotypically plastic adjustment of traits such as behaviour, morphology and life-history (West-Eberhard, 2003; DeWitt & Scheiner, 2004). To be able to respond quickly and efficiently to threats *via* such inducible defences, organisms need to continuously monitor their environment and assess predation risk. In case of animals forming groups either for foraging or to avoid predation, individuals also need to consider the size of the assemblage to correctly estimate predation risk and mount a cost-effective response. This is because the *per capita* predation risk may be inversely related to the size of the group, due to the decrease in chance of predation (dilution effect), increased vigilance (the "many eyes" effect) or predator confusion (Pulliam, 1973; Elgar, 1989; Lima, 1995; Roberts, 1996; Peacor, 2003). Therefore, when the *per capita* predation risk is lower at higher conspecific densities, individuals should invest less in costly plastic anti-predator responses (Peacor, 2003). In line

with this theory, empirical studies on several taxa demonstrated that prey individuals adjust their morphological and behavioural anti-predator defences to high conspecific density by producing less intense responses to predation risk (McCoy, 2007; Van Buskirk et al., 2011; Tollrian et al., 2015), although the effect of conspecific density on anti-predator responses may change during ontogeny, at least in part due to changes in predation risk (Davenport & Chalcraft, 2014).

Aggregations may provide protection against predation, but exposure to high densities of conspecifics can also entail costs, arising from increased resource competition (Morin, 1986; Holbrook & Schmitt, 2002; Hixon & Jones, 2005; Amundsen et al., 2007), cannibalism (Wildy et al., 2001; Jordan et al., 2004; Jefferson et al., 2014; DeVore et al., 2021), or facilitated spread of pathogens (Smith et al., 2009; Briggs et al., 2010; Eskew & Todd, 2013; Sanchez & Hudgens, 2019; Malagon et al., 2020) and parasites (Arneberg et al., 1998; Morand & Poulin, 1998; Lindsey et al., 2009). Limited resources allocated to preventing or combating these negative effects of group living may be traded-off against antipredator-responses. Furthermore, anti-predator responses may be also weakened when a phenotype beneficial against conspecifics or infections is disadvantageous against predators. For example, certain phenotypic changes in amphibian larvae, like higher foraging activity, a longer body and a shallower tail, benefit fitness in the presence of competitors, but the same changes are maladaptive in the presence of certain predators (Relyea, 2002; Relyea, 2004; Relyea & Auld, 2005). Examples of such conflicts among responses induced by different enemies are abundant (e.g. Sih et al., 1998; DeWitt et al., 2000; Teplitsky et al., 2004).

Conflict between anti-predator and anti-competitor defences is, however, not inevitable, because a single response may provide protection both against predators and the perils of aggregations. Chemical defence, i.e. production of toxic or noxious compounds against enemies, often represents such a multifunctional response (Izhaki, 2002; Apponyi et al., 2004; Holopainen, 2004; Thoms & Schupp, 2007; Núñez-Pons et al., 2012; Gasch et al., 2013; Schierling et al., 2013). For example, toxins of toads (Bufonidae, Amphibia) deter several predators (e.g. Henrikson, 1990; Greenlees et al., 2010; Üveges et al., 2019), but they also have antibacterial (Cunha Filho et al., 2005), antifungal (Barnhart et al., 2017) and antiparasitic properties (Tempone et al., 2008). Also, the cell type (giant cells; Riesenzellen) associated with toxin synthesis in toad tadpoles (Delfino et al., 1995; Regueira et al., 2016) was suggested to be the source of allelochemical agents that inhibit the growth of conspecifics (Crossland & Shine, 2012; Clarke et al., 2015). In line with this potential of toad toxins for providing protection from multiple enemies, it has also been shown that larvae and juveniles of toads increase their toxin production in response to predation risk (Benard & Fordyce, 2003; Hagman et al., 2009; Hettyey et al., 2019) and high conspecific density (Bókony et al., 2018).

When the same phenotype is beneficial against both predators and the negative effects of group living, investment in such a multifunctional defence is expected to respond differently to the interplay between predation risk and conspecific density than when the anti-predator and anti-group responses are in conflict (Fig. 1; Poitrineau et al., 2003). When the anti-predator defence is in trade-off with the anti-group defences (Fig. 1a), expression of the anti-predatory trait should increase with increasing predation risk and decrease with increasing conspecific density (Peacor, 2003; McCoy, 2007; Van Buskirk et al., 2011; Tollrian et al., 2015). These two effects were usually found to be additive (Van Buskirk et al., 2011; Tollrian et al., 2015). In contrast, when the same induced response is effective against both predators and competitors, exposure to both types of enemies should result in enhanced responses (Fig. 1b). However, to alleviate costs due to physiological constraints or energetic trade-offs (Blennerhassett et al., 2019), the optimal strategy for animals with such defences should be to "kill two birds with one stone". That is, they should dampen their response to an

enemy if their defence level is already so high, due to their response to other enemies, that a further induction of toxin synthesis by the enemy in question no longer provides additional fitness benefits (Poitrineau et al., 2003). Therefore, we expect the effects of the two enemies to be non-additive: a combination of high predation risk and high conspecific density may induce only slightly higher investment into defence than either one of these factors alone (Fig. 1b). However, in the case of animals, we know of no study that has investigated the interaction between the effects of predation risk and conspecific density on inducible chemical defences that are effective against both predators and the dangers posed by conspecifics.

We investigated the combined effects of conspecific density and predation risk on inducible chemical defence by conducting a mesocosm experiment in which we reared common toad *Bufo bufo* tadpoles at three conspecific densities in the presence or absence of chemical cues indicating predation risk. Tadpoles of this species regularly form aggregations (Watt et al., 1997; Griffiths & Foster, 1998) and synthesise toxins from an early age (Üveges et al., 2017). The main defensive compounds of toads are cardiotoxic steroids called bufadienolides (Toledo & Jared, 1995; Krenn & Kopp, 1998; Gao et al., 2010) that are distasteful, poisonous or even deadly to predators (Greenlees et al., 2010; Somaweera et al., 2011; Chen & Huang, 2013). A previous experiment showed that common toad tadpoles increase their bufadienolide synthesis in response to high conspecific density in the absence of predators (Bókony et al., 2018). Also, tadpoles raised in groups did not change their toxin production in response to chemical cues indicating predation risk in two other studies (Üveges et al., 2017; Üveges et al., 2019). However, when tadpoles were kept individually in a fourth experiment, toxin synthesis was enhanced upon exposure to chemical cues indicating predation risk (Hettyey et al., 2019). Together, these results suggest that the effect of predators on toxin production of common toad tadpoles may depend on conspecific density. Therefore, we predicted a non-additive effect when tadpole density and predation risk are manipulated simultaneously (Fig. 1), i.e. that the difference in bufadienolide content between tadpoles raised with and without cues indicating predation risk should diminish with increasing conspecific density.

Materials and methods
Experimental Procedures
In spring 2018, we collected 140 eggs from each of six freshly laid common toad clutches from a pond in the Pilis Mountains, Hungary (Szárazfarkas-belső; 47°44'4.12"N, 18°49'7.04"E). We also collected 120 eggs from each of ten clutches of agile frogs *Rana dalmatina* from the same pond to be later used as food for predators (see below). We transported eggs to the laboratory of the Plant Protection Institute, Centre for Agricultural Research (Budapest, Hungary), and kept each family until hatching in 1 L reconstituted soft water (RSW, 48 mg×L^{-1} NaHCO$_3$, 30 mg×L^{-1} CaSO$_4$ × 2 H$_2$O, 61 mg×L^{-1} MgSO$_4$ × 7 H$_2$O, 2 mg×L^{-1} KCl added to reverse-osmosis filtered, UV-sterilized tap water). After hatching, we kept each family of tadpoles in 5 L RSW in the laboratory until they reached the free-swimming stage (developmental stage 25, Gosner, 1960). During this part of the experiment, tadpoles developed at 21 °C ambient temperature and a 13:11 hours light:dark cycle.

Three weeks before the start of the experiment, we set up 48 outdoor mesocosms by filling plastic containers (57 × 39 × 28 cm, length × width × height) with 40 L aged tap water, inoculating them with 0.6 L pond water containing algae and zooplankton, and adding 20 g dried beech *Fagus sylvatica* leaves into each container. This ensured food availability due to algal growth, and provided refugia for tadpoles. During the course of the study overflow holes in the wall of plastic containers kept the water levels from rising. To prevent colonization of mesocosms by invertebrate predators, we covered containers with mosquito net lids. When

toad tadpoles reached the free-swimming stage, we introduced them into the mesocosms and raised them for the treatment period. We kept remaining toad tadpoles and all agile frog tadpoles in additional mesocosms (82 × 58 × 30 cm) filled with 130 L aged tap water.

To test the effects of conspecific density and predation risk on induced chemical defences, we applied a factorial experimental design. We transferred one, two or four haphazardly selected toad tadpoles from each family into each mesocosm, resulting in six, twelve or twenty four tadpoles per mesocosm, which represented low, medium and high tadpole densities, respectively (Bókony et al., 2018), and we assigned each mesocosm to one of two predator treatments: cues present or absent. We replicated each of the six treatment combinations eight times, resulting in 48 experimental units (three densities of conspecifics × two predator treatments × eight replicates). We arranged treatments in a randomized block design, where each of the eight blocks contained one mesocosm from each treatment combination.

Chemical cues indicating predation risk were provided by eight adult European perch *Perca fluviatilis* which were kept together in a tank (82 × 58 × 30 cm) containing 130 L aerated aged tap water. Fishes are widely regarded as the most dangerous predators to tadpoles in general (Wells, 2007) and, compared to newts and dragonfly larvae, perch elicited the strongest response in the chemical defence of toad tadpoles in a previous experiment (Hettyey et al., 2019). Fish weighed in total 336.46 g at the beginning and 290.77 g at the termination of the experiment. Fish were fed daily with 6.05 ± 0.04 g (mean ± SD) agile frog larvae and 0.61 ± 0.11 g common toad tadpoles. Fish always ate all agile frog tadpoles and killed 40.09 ± 27.02 % of toad tadpoles that were offered as food (mean ± SD; of all offered toad tadpoles 35.17 ± 25.05 % were consumed and 4.92 ± 7.19 % were killed but not consumed).

We created stimulus water by homogenizing 1885.67 ± 6.92 mg toad tadpoles with a blender in ca. 50 ml RSW and adding this homogenate to 25 L water taken from the fish tank (Benard & Fordyce, 2003; Hettyey et al., 2019). We repeated this process daily 2-3 hours after feeding the fish and subsequently refilled the fish tank to the original volume using aged tap water. The addition of the tadpole homogenate was necessary to ensure that experimental tadpoles were exposed to sufficiently high concentrations of prey-borne cues of predation even when fish did not eat all toad tadpoles, because conspecific alarm cues are required for eliciting strong antipredator responses (Laurila et al., 1997; Schoeppner & Relyea, 2005; Hettyey et al., 2015).

After thoroughly mixing the stimulus water, we poured 800 ml of the mixture into each mesocosm assigned to the predator treatment, and 800 ml of aged tap water into each mesocosm holding control tadpoles (i.e. those assigned to the treatment groups without cues indicating predation risk). As a result, experimental tadpoles were exposed to chemical cues corresponding to 48.25 ± 4.97 mg×L^{-1} fish (kairomones, mean ± SD), 0.86 ± 0.14 mg×L^{-1} heterospecifics and a maximum of 0.09 ± 0.01 mg×L^{-1} conspecifics (alarm pheromones ("Schreckstoff", von Frisch, 1942), and chemical cues released via the digestion of tadpoles), as well as to 1.51 ± 0.01 mg×L^{-1} homogenized conspecifics (cues released by mechanical damage). Similar cue concentrations elicited clear antipredator responses in chemical defences of common toad tadpoles in a previous study (Hettyey et al., 2019).

We terminated the experiment after two weeks of treatment, when most of the experimental tadpoles reached developmental stage 36 (Gosner, 1960). We chose this time frame because bufadienolide content of common toads peaks around this stage during their larval development (Ujszegi et al., 2017; Üveges et al., 2017). We haphazardly selected six tadpoles from each mesocosm and preserved them in HPLC-grade absolute methanol for chemical analysis (n = 288). We randomly selected three methanol-preserved tadpoles from each tub (n = 144) and assessed their developmental stage according to Gosner (1960) using a

stereomicroscope. Developmental stage of tadpoles was highly uniform (stage 35: n = 10, stage 36: n = 134) and similarly distributed across all six treatment-combinations (Fisher's exact test, P = 0.229). No experimental animals died before the termination of treatments, and after the experiment we released all remaining tadpoles into their pond of origin.

Chemical Analysis

We prepared samples by homogenising preserved tadpoles using a VWR VDI 12 homogenizer with an IKA S12N-7S dispersing tool. Subsequently, we dried homogenates *in vacuo* at 45 C° using a Büchi Rotavapor R-134 rotary evaporator and measured dry mass to the nearest 0.1 mg with an analytical balance (Sartorius Entris 224i-1S). Samples were re-dissolved in 1 ml HPLC-grade absolute methanol, facilitated by brief exposure to ultrasound in a Tesla UC005AJ1 bath sonicator. Finally, we filtered samples using FilterBio nylon syringe filters (pore size = 0.22 μm).

We analysed samples 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 & Fordyce, 2003; Hagman et al., 2009; Üveges et al., 2017; Bókony et al., 2018; Hettyey et al., 2019; Üveges et al., 2019) and by co-injection with standards of the following bufadienolides: bufalin, bufotalin, resibufogenin, gamabufotalin, areno- and telocinobufagin (Biopurify Phytochemicals, Chengdu, China), cinobufagin (Chembest, Shanghai, China), cinobufotalin (Quality Phytochemicals, New Jersey, USA), digitoxigenin (Santa Cruz Biotechnology, Dallas, TX, USA) and marinobufotoxin (courtesy of Dr. Rob Capon, University of Queensland, Brisbane, Australia). Furthermore, to help identify bufadienolide compounds present in low quantities, we analysed a bulk sample obtained from 49 juvenile common toads by manually applying pressure to their parotoid glands.

We quantified bufadienolide compounds using a single-quadrupole HPLC-MS system (Model LC-MS-2020, Shimadzu, Kyoto, Japan) equipped with a binary gradient solvent pump, a vacuum degasser, a thermostated autosampler, a column oven, a photodiode detector and a mass analyzer with electrospray ionization (ESI/MS). Ten μl of samples were injected at 35 °C on a Kinetex C18 2.6 μm column (100 mm x 3 mm i.d.) in series with an octadecyl C_{18} guard column (4 mm × 3 mm i.d.). Eluent A was 5 % aqueous acetonitrile with 0.05 % formic acid, eluent B was acetonitrile with 0.05 % formic acid. The flow rate was 0.6 ml/min and the gradient was as follows: 0-1 min: 10-20 % B; 1-11 min: 20-29 % B; 11-13 min: 29-58 % B; 13.1-16 min: 100 % B; 16.1-20 min: 10 % B. ESI conditions were set as follows: interface temperature: 350 °C; desolvation line (DL) temperature: 250 °C; heat block temperature: 400 °C; drying N_2 gas flow: 15 L×min^{-1}; nebulizer N_2 gas flow: 1.5 L×min^{-1}; positive ionization mode. Full scan spectra were recorded in the range of 350–800 m/z and we also performed selected-ion monitoring (SIM) detecting the base peaks of bufadienolides we previously found in common toads (Üveges et al., 2017; Bókony et al., 2018; Hettyey et al., 2019). Data were processed using the LabSolutions 5.42v software (Shimadzu, Kyoto, Japan).

Statistical Analysis

We used total bufadienolide quantity (TBQ), mass-corrected total bufadienolide quantity (mcTBQ) and the number of bufadienolide compounds (NBC) to analyse toxin content of toad tadpoles. We calculated TBQ and NBC from MS chromatogram peaks. 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., 2019). We estimated the quantity of each compound from the area values of chromatogram peaks based on the calibration curve of the marinobufotoxin standard. 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, and was used also in former studies (e.g. Benard & Fordyce, 2003; Hagman et al., 2009; Üveges et al., 2017; Bókony et al., 2018; Hettyey et al., 2019; Üveges et al., 2019). We subsequently summed these values to obtain estimates of TBQ for each individual. We divided TBQ by the dry mass of samples to get mass-corrected total bufadienolide quantity (mcTBQ). TBQ measures the total toxin quantity of tadpoles, which is likely to be relevant for the efficacy of the chemical defence, whereas mcTBQ reflects the relative amount of resources allocated to chemical defence. Although the bufadienolide quantity of the skin is the most relevant for predator deterrence, we did not estimate body surface area because it is strongly correlated with body mass in wood frog *Rana sylvatica* tadpoles (Davis et al., 2008) and we did not expect body shape differences between treatment groups due to the low morphological plasticity of common toad larvae (Lardner, 2000; Van Buskirk, 2002; Van Buskirk, 2009; Üveges et al., 2019). Finally, NBC is a measure of diversity of the toxin cocktail produced by individual tadpoles, which may be relevant for protection from multiple threats (i.e. different toxin compounds may be effective against different enemies).

Statistical analyses were run in R 3.4.0 (R Development Core Team, 2017). We used linear mixed-effects models (LMM), implemented with the 'lme' function in 'nlme' (Pinheiro et al., 2017), entering TBQ, mcTBQ, NBC or dry mass as the dependent variable, predator treatment, conspecific density, and their interaction as fixed factors, and mesocosm as random factor. Preliminary likelihood-ratio tests indicated that block as random factor had no effect, therefore it was omitted from the analyses. In the models of mcTBQ and dry mass, we included the 'weights' argument with the 'varIdent' function to account for differences in variances between the six treatment combinations and to improve model fit. We obtained *P*-values for each model term (the two main effects and their interaction) from type-2 analysis-of-deviance tables using the 'Anova' function of the 'car' package (Fox & Weisberg, 2019). To test our predictions, we conducted planned comparisons (Ruxton & Beauchamp, 2008; Chen et al., 2018) using linear contrasts for each of our dependent variables calculated from our LMM models, similarly to Hettyey et al. (2019). First, we tested whether density of conspecifics affected the dependent variables by comparing the estimated marginal means pairwise between the three density treatments. We performed these tests as averaged for the two predator treatment groups (controls and tadpoles exposed to cues indicating predation risk) and also within each predator treatment group. Second, we also tested whether the predator treatment affected the dependent variables within each density group. Finally, we tested whether the effect of cues indicating predation risk varied with tadpole density, by comparing the anti-predator response (i.e. the estimated difference between the tadpoles reared in the presence and absence of cues indicating predation risk at each tadpole density) pairwise between the three density treatments, and also between the two lowest densities *versus* the highest density of conspecifics. We calculated linear contrasts with the 'emmeans' package (Lenth et al., 2019), and applied the FDR (false-discovery rate) method to adjust *P*-values for multiple comparisons (Benjamini & Hochberg, 1995; Pike, 2011). For the annotated R script of the statistical analysis, see the Supporting Information.

Results
Tadpoles in the high density treatment exhibited significantly decreased body mass compared to the two lower densities of tadpoles, and exposure to chemical cues indicating predation risk resulted in significantly decreased tadpole body mass compared to control tadpoles at medium and low densities (Tables S1-S4, Fig. S1). Despite these differences, toxin content did not decrease either with high conspecific density or under predation risk. Total bufadienolide quantity (TBQ) of tadpoles reared at high density was significantly higher than at medium density, while TBQ did not differ between high and low density and between the two lower

densities (Table 1, S2 & S5, Fig. 2; note however the marginally non-significant difference between the high and low densities). When analysed within density treatments, TBQ of tadpoles did not differ significantly between predator treatments (Table S3, Fig. 2 & S2) despite an overall tendency for higher TBQ in response to cues indicating predation risk (Table 1 & S3, Fig. 2). This slight response to cues indicating predation risk on TBQ did not vary significantly with tadpole density (Table 1, Table S4, Fig. 2 & S2).

Similarly to TBQ, mass-corrected total bufadienolide quantity (mcTBQ) of tadpoles was also significantly higher at high conspecific density than at medium and low densities, and did not differ between the two lower densities (Table 1 & S2, Fig. 2). In contrast to TBQ, however, tadpoles that received chemical cues indicating predation risk had significantly higher mcTBQ compared to their control conspecifics at both low and medium densities, and there was a similar but non-significant tendency when density was high (Table S3, Fig. 2 & S2). The response to predation risk in mcTBQ did not vary significantly with tadpole density (Table 1, Table S4, Fig. S2).

The number of bufadienolide compounds (NBC) was not affected either by different levels of conspecific density or by the presence or absence of chemical cues indicating predation risk (Table 1 & S1-S4, Fig. 2 & S2).

Discussion

Both high conspecific density and exposure to chemical cues indicating predation risk can induce a plastic increase in the toxin synthesis of toad tadpoles, as can be expected of a defence effective against multiple enemies (Bókony et al., 2018; Hettyey et al., 2019). The present study shows that plastic responses in chemical defence induced by conspecific density and by cues indicating predation risk are expressed simultaneously. That is, high conspecific density increases toxin content not only in a predator-free environment (Bókony et al., 2018), but also in the presence of predators (Table S2), and similarly, the presence of chemical cues of predation risk increases investment into toxin production not only in isolated tadpoles (as shown by Hettyey et al., 2019), but also in groups, at least at low and medium densities of conspecifics.

In agreement with our prediction (Fig. 1b), we found that the effect of predation risk on toxin content was no longer significant at the highest conspecific density, suggesting that the effects of predators and conspecifics may become non-additive with increasing conspecific densities. At the same time, however, the interaction between predator treatment and conspecific density was not significant, i.e. the intensity of the antipredator response in toxin synthesis did not differ significantly between density treatments. This latter result does not support non-additive effects, suggesting instead that the effects of predation risk and conspecific density on toxin synthesis may simply be additive. This complexity of our results is apparently due to relatively small effect sizes coupled with relatively high variability (Fig. 2). The anti-predatory response in mcTBQ at high conspecific density was only 72 % of the average response seen at the two lower densities (Table S4), which seems a biologically relevant difference. However, there was high variance between responses of tadpoles especially at high density, resulting in largely overlapping ranges of anti-predatory responses at all densities (Fig. S2). Altogether, these findings suggest that the high toxin levels induced by high conspecific density might lead to reduced further increases in toxin investment in response to predation risk, but this reduction was very small in our study. It is possible that the effects of predators and conspecifics on chemical defence are additive at certain levels and non-additive at other levels of predation risk and conspecific density. Exploring this possibility in future studies could yield valuable insights into the functional ecology of inducible defences that are effective against multiple enemies.

8

The higher bufadienolide content of tadpoles at high conspecific density aligns well with the notion that this chemical defence is likely effective against multiple enemies. Enhanced bufadienolide content may benefit toad tadpoles exposed to high densities of conspecifics in several ways. Theoretically, toxins may be utilized against competitors as allelochemicals to reduce their growth (Crossland & Shine, 2012; Clarke et al., 2015) or as a defence against cannibalistic attempts (Wildy et al., 2001; Jordan et al., 2004; Jefferson et al., 2014). However, toads appear to be resistant to the toxic effects of bufadienolides (Moore et al., 2009; Crossland et al., 2011; Crossland & Shine, 2011; DeVore et al., 2021). For example, in cane toad *Rhinella marina* tadpoles, bufadienolides do not deter, but rather attract cannibalistic conspecifics (Crossland et al., 2012), so that cannibals can devour up to 99.9 % of hatchling conspecifics (DeVore et al., 2021). Therefore, bufadienolides may not provide an effective defence against attacks from conspecific tadpoles (DeVore et al., 2021). It is more likely that toad toxin production is induced in response to high conspecific densities because bufadienolides may mitigate infection risk by inhibiting the growth of pathogenic bacteria (Cunha Filho et al., 2005), the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Barnhart et al., 2017) and endoparasitic protozoans (Tempone et al., 2008).

The other major function of bufadienolides is anti-predatory protection (Toledo & Jared, 1995; Greenlees et al., 2010; Llewelyn et al., 2012; Üveges et al., 2019). For anti-predatory defences, the pay-off of investment is expected to decrease with increasing conspecific density (Fig. 1a), because the benefits diminish as a consequence of reduced *per capita* predation risk (Peacor, 2003; Van Buskirk et al., 2011), whereas the costs increase due to intensifying competition for resources, and/or because physiology may set an upper limit to defence expression. This has been supported by several empirical studies on density dependence of behavioural and morphological anti-predator responses of different animal species (Wiackowski & Starońska, 1999; Relyea & Hoverman, 2003; Relyea, 2004; McCoy, 2007; Van Buskirk et al., 2011; Davenport & Chalcraft, 2014; Tollrian et al., 2015). However, for defences that provide protection against multiple types of enemies, the effects of high conspecific density on antipredator defences may be different (Fig. 1b), similar to the synergy proposed between defensive traits that provide cross-resistance against multiple enemies (Poitrineau et al., 2003). Since toad tadpoles exposed to cues indicating predation risk increased rather than decreased their bufadienolide content with increasing conspecific density, our findings suggest that the density-dependence of toxin production was more strongly affected by the need for protection against the negative effects of high conspecific density than by the positive effects of group size on anti-predatory protection (such as risk dilution). Notably, the high density treatment in our study was not extreme compared to naturally occurring densities of toad tadpoles (Arnold & Wassersug, 1978; Bókony et al., 2016; B. Üveges pers. obs.). Consequently, it is possible that the *per capita* predation risk perceived by tadpoles in our experiment was not low enough to make a decrease in anti-predator chemical defence pay off, nor to make a further increase in response to predation risk impossible due to physiological limits. Thus, it remains to be tested if tadpole densities higher than those applied in this study would result in greatly reduced anti-predatory responses in terms of bufadienolide synthesis.

The number of bufadienolide compounds present in tadpoles was not affected either by conspecific density or by predation risk (similar to Üveges et al., 2017; Bókony et al., 2018). Although an earlier study found inducible changes in bufadienolide compound diversity as a response to predators (Hettyey et al., 2019), the magnitude of that change was small. It is possible that toxin cocktail diversity is a less plastic trait than toxin amount, or the plastic response in compound diversity may be relatively difficult to detect, perhaps because cocktail diversity may be confounded by bacterial transformation of bufadienolide compounds (Hayes et al., 2009; Kamalakkannan et al., 2017). Currently, the relative effects of

each bufadienolide compound on natural enemies are barely known (Chen & Chen, 1933; Cunha Filho et al., 2005; Tempone et al., 2008; Crossland et al., 2012; Barnhart et al., 2017), despite the possibility that different enemies might be sensitive to different compounds. Thus, the functional importance of cocktail diversity in chemical defences remains to be tested.

The observed decrease in body mass in response to increasing conspecific density and to the presence of cues indicating predation risk (Fig. S1) aligns well with previous studies and is likely a consequence of competition for food and reduced activity in response to predation risk (e.g. Skelly & Werner, 1990; Werner & Anholt, 1993; Laurila et al., 1998). This decrease in body mass may have arisen, at least in part, as a cost of higher investment into toxin production at high densities and under predation risk, because such investment can interfere with energy metabolism and growth (Blennerhassett et al., 2019). However, this scenario seems unlikely in our case, because there seems to be no systematic relationship between body mass and total bufadienolide quantity within treatment groups in our experiment (Fig. S3) and previous studies also did not find considerable costs of toxin synthesis in common toad tadpoles (Kurali et al., 2016; Üveges et al., 2017).

Lastly, our results may also have implications for conservation biology. Invasive toad species, such as the cane toad in Australia (Shine, 2010), and the Asian common toad *Duttaphrynus melanostictus* in Madagascar (Licata et al., 2019) pose serious threats to the native fauna, mainly due to their toxicity. If the results of our study are applicable to these toad species, removal efforts focusing on early-stage tadpoles may be beneficial not only by decreasing the number of toads in invaded regions, but also by decreasing the toxin content of their tadpoles (which might also have long-lasting effects on their toxicity after metamorphosis; see Benard & Fordyce, 2003; Hagman et al., 2009). Lower toxicity of toads may prevent mortality of native predators due to poisoning and may allow them to learn to avoid toxic invaders, thereby facilitating adaptation of the local predator fauna (Phillips & Shine, 2006; Greenlees et al., 2010; Caller & Brown, 2013). Therefore, information on how chemically defended invaders adjust their toxin production to environmental conditions may help efforts focusing on their management and the protection of native species.

References

Amundsen, P.-A., Knudsen, R. & Klemetsen, A. (2007). Intraspecific competition and density dependence of food consumption and growth in Arctic charr. *Journal of Animal Ecology, 76,* 149-158. https://doi.org/10.1111/j.1365-2656.2006.01179.x

Apponyi, M. A., Pukala, T. L., Brinkworth, C. S., Maselli, V. M., Bowie, J. H., Tyler, M. J., Booker, G. W., Wallace, J. C., Carver, J. A., Separovic, F., Doyle, J. & Llewellyn, L. E. (2004). Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance. *Peptides, 25,* 1035-1054. https://doi.org/10.1016/j.peptides.2004.03.006

Arneberg, P., Skorping, A., Grenfell, B. & Read, A. F. (1998). Host densities as determinants of abundance in parasite communities. *Proceedings of the Royal Society B: Biological Sciences, 265,* 1283-1289. https://doi.org/10.1098/rspb.1998.0431

Arnold, S. J. & Wassersug, R. J. (1978). Differential predation on metamorphic anurans by garter snakes (*Thamnophis*): social behavior as a possible defense. *Ecology, 59,* 1014-1022. https://doi.org/10.2307/1938553

Barnhart, K., Forman, M. E., Umile, T. P., Kueneman, J., McKenzie, V., Salinas, I., Minbiole, K. P. C. & Woodhams, D. C. (2017). Identification of bufadienolides from the boreal toad, *Anaxyrus boreas*, active against a fungal pathogen. *Microbial Ecology, 74,* 990-1000. https://doi.org/10.1007/s00248-017-0997-8

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

Benjamini, Y. & Hochberg, Y. (1995). Controlling 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. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Blennerhassett, R. A., Bell-Anderson, K., Shine, R. & Brown, G. P. (2019). The cost of chemical defence: the impact of toxin depletion on growth and behaviour of cane toads (*Rhinella marina*). *Proceedings of the Royal Society B: Biological Sciences, 286,* 20190867. https://doi.org/10.1098/rspb.2019.0867

Bókony, V., Móricz, Á. M., Tóth, Z., Gál, Z., Kurali, A., Mikó, Z., Pásztor, K., Szederkényi, M., Tóth, Z., Ujszegi, J., Üveges, B., Krüzselyi, D., Capon, R. J., Hoi, H. & Hettyey, A. (2016). Variation in chemical defense among natural populations of common toad, *Bufo bufo*, tadpoles: the role of environmental factors. *Journal of Chemical Ecology, 42,* 329-338. https://doi.org/10.1007/s10886-016-0690-2

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

Briggs, C. J., Knapp, R. A. & Vredenburg, V. T. (2010). Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences, 107,* 9695-9700. https://doi.org/10.1073/pnas.0912886107

Caller, G. & Brown, C. (2013). Evolutionary responses to invasion: Cane toad sympatric fish show enhanced avoidance learning. *PLoS ONE, 8,* e54909-e54909. https://doi.org/10.1371/journal.pone.0054909

Chen, K. K. & Chen, A. L. (1933). Similarity and dis-similarity of bufagins, bufotoxins, and digitaloid glucosides. *Journal of Pharmacology and Experimental Therapeutics, 49,* 561-579.

Chen, L. & 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. https://doi.org/10.1016/j.forsciint.2013.08.013

Chen, T., Xu, M., Tu, J., Wang, H. & Niu, X. (2018). Relationship between omnibus and post-hoc tests: An investigation of performance of the F test in ANOVA. *Shanghai Archives of Psychiatry, 30,* 60-64. https://doi.org/10.11919/j.issn.1002-0829.218014

Clarke, G. S., Crossland, M. R., Shilton, C. & Shine, R. (2015). Chemical suppression of embryonic cane toads *Rhinella marina* by larval conspecifics. *Journal of Applied Ecology, 52,* 1547-1557. https://doi.org/10.1111/1365-2664.12528

Crossland, M. R., Haramura, T., Salim, A. A., Capon, R. J. & Shine, R. (2012). Exploiting intraspecific competitive mechanisms to control invasive cane toads (Rhinella marina). *Proceedings of the Royal Society B: Biological Sciences, 279,* 3436-3442. https://doi.org/10.1098/rspb.2012.0821

Crossland, M. R., Hearnden, M. N., Pizzatto, L., Alford, R. A. & Shine, R. (2011). Why be a cannibal? The benefits to cane toad, *Rhinella marina* [=*Bufo marinus*], tadpoles of consuming conspecific eggs. *Animal Behaviour, 82,* 775-782. https://doi.org/10.1016/j.anbehav.2011.07.009

Crossland, M. R. & Shine, R. (2011). Cues for cannibalism: cane toad tadpoles use chemical signals to locate and consume conspecific eggs. *Oikos, 120,* 327-332. https://doi.org/10.1111/j.1600-0706.2010.18911.x

Crossland, M. R. & Shine, R. (2012). Embryonic exposure to conspecific chemicals suppresses cane toad growth and survival. *Biology Letters, 8,* 226-229. https://doi.org/10.1098/rsbl.2011.0794

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. & Schwartz, E. F. (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. https://doi.org/10.1016/j.toxicon.2005.01.017

Davenport, J. M. & Chalcraft, D. R. (2014). Increasing conspecific density weakens the ability of intermediate predators to develop induced morphological defences to top predators. *Freshwater Biology, 59,* 87-99. https://doi.org/10.1111/fwb.12248

Davis, A. K., Connell, L. L., Grosse, A. & Maerz, J. C. (2008). A fast, non-invasive method of measuring growth in tadpoles using image analysis. *Herpetological Review, 39,* 56-57.

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

DeVore, J. L., Crossland, M. R. & Shine, R. (2021). Tradeoffs affect the adaptive value of plasticity: stronger cannibal-induced defenses incur greater costs in toad larvae. *Ecological Monographs, 91,* e01426. https://doi.org/10.1002/ecm.1426

DeWitt, T. J., Robinson, B. W. & Wilson, D. S. (2000). Functional diversity among predators of a freshwater snail imposes an adaptive trade-off for shell morphology. *Evolutionary Ecology Research, 2,* 129-148.

DeWitt, T. J. & Scheiner, S. M. (2004). Phenotypic plasticity: functional and conceptual approaches. Oxford University Press.

Elgar, M. A. (1989). Predator vigilance and group size in mammals and birds: A critical review of the empirical evidence. *Biological Reviews, 64,* 13-33. https://doi.org/10.1111/j.1469-185X.1989.tb00636.x

Eskew, E. A. & Todd, B. D. (2013). Parallels in amphibian and bat declines from pathogenic fungi. *Emerging Infectious Diseases, 19,* 379-385. https://doi.org/10.3201/eid1093.120707

Fox, J. & Weisberg, S. (2019). An R Companion to Applied Regression (3 ed.). Sage Publications.

Gao, H., Zehl, M., Leitner, A., Wu, X., Wang, Z. & Kopp, B. (2010). Comparison of toad venoms from different *Bufo* species by HPLC and LC-DAD-MS/MS. *Journal of Ethnopharmacology, 131,* 368-376. https://doi.org/10.1016/j.jep.2010.07.017

Gasch, T., Schott, M., Wehrenfennig, C., Düring, R.-A. & Vilcinskas, A. (2013). Multifunctional weaponry: The chemical defenses of earwigs. *Journal of Insect Physiology, 59,* 1186-1193. https://doi.org/10.1016/j.jinsphys.2013.09.006

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

Greenlees, M. J., Phillips, B. L. & Shine, R. (2010). Adjusting to a toxic invader: Native Australian frogs learn not to prey on cane toads. *Behavioral Ecology, 21,* 966-971. https://doi.org/10.1093/beheco/arq095

Griffiths, R. A. & Foster, J. P. (1998). The effect of social interactions on tadpole activity and growth in the British anuran amphibians (*Bufo bufo*, *B. calamita*, and *Rana temporaria*). *Journal of Zoology, 245,* 431-437. https://doi.org/10.1111/j.1469-7998.1998.tb00117.x

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

Hayes, R. A., Piggott, A. M., Dalle, K. & Capon, R. J. (2009). Microbial biotransformation as a source of chemical diversity in cane toad steroid toxins. *Bioorganic & Medicinal Chemistry Letters, 19,* 1790-1792. https://doi.org/10.1016/j.bmcl.2009.01.064

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

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

Hettyey, A., Üveges, B., Móricz, Á. M., Drahos, L., Capon, R. J., Van Buskirk, J., Tóth, Z. & Bókony, V. (2019). Predator-induced changes in the chemical defence of a vertebrate. *Journal of Animal Ecology, 00,* 1-12. https://doi.org/10.1111/1365-2656.13083

Hixon, M. A. & Jones, G. P. (2005). Competition, predation, and density-dependent mortality in demersal marine fishes. *Ecology, 86,* 2847-2859. https://doi.org/10.1890/04-1455

Holbrook, S. J. & Schmitt, R. J. (2002). Competition for shelter space causes density-dependent predation mortality in damselfishes. *Ecology, 83,* 2855-2868. https://doi.org/10.1890/0012-9658(2002)083[2855:cfsscd]2.0.co;2

Holopainen, J. K. (2004). Multiple functions of inducible plant volatiles. *Trends in Plant Science, 9,* 529-533. https://doi.org/10.1016/j.tplants.2004.09.006

Izhaki, I. (2002). Emodin – a secondary metabolite with multiple ecological functions in higher plants. *New Phytologist, 155,* 205-217. https://doi.org/10.1046/j.1469-8137.2002.00459.x

Jefferson, D. M., Hobson, K. A. & Chivers, D. P. (2014). Time to feed: How diet, competition, and experience may influence feeding behaviour and cannibalism in wood frog tadpoles *Lithobates sylvaticus*. *Current Zoology, 60,* 571-580. https://doi.org/10.1093/czoolo/60.5.571

Jordan, D. J., Rombough, C. J., Pearl, C. A. & McCreary, B. (2004). Cannibalism and predation by western toad (*Bufo boreas boreas*) larvae in Oregon, USA. *Western North American Naturalist, 64,* 403-405.

Kamalakkannan, V., Salim, A. A. & Capon, R. J. (2017). Microbiome-mediated biotransformation of cane toad bufagenins. *Journal of Natural Products, 80,* 2012-2017. https://doi.org/10.1021/acs.jnatprod.7b00134

Krenn, L. & Kopp, B. (1998). Bufadienolides from animal and plant sources. *Phytochemistry, 48,* 1-29. https://doi.org/10.1016/S0031-9422(97)00426-3

Kurali, A., Pásztor, K., Hettyey, A. & Tóth, Z. (2016). Toxin depletion has no effect on antipredator responses in common toad (*Bufo bufo*) tadpoles. *Biological Journal of the Linnean Society, 119,* 1000-1010. https://doi.org/10.1111/bij.12864

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

Laurila, A., Kujasalo, J. & Ranta, E. (1997). Different antipredator behaviour in two anuran tadpoles: effects of predator diet. *Behavioral Ecology and Sociobiology, 40,* 329-336. https://doi.org/10.1007/s002650050349

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

Lenth, R. V., Singmann, H., Love, J., Buerkner, P. & Herve, M. (2019). emmeans: Estimated marginal means, aka least-squares means. *CRAN,* https://doi.org/10.1080/00031305.1980.10483031

Licata, F., Ficetola, G. F., Freeman, K., Mahasoa, R. H., Ravololonarivo, V., Solofo Niaina Fidy, J. F., Koto-Jean, A. B., Nahavitatsara, E. R., Andreone, F. & Crottini, A. (2019). Abundance, distribution and spread of the invasive Asian toad *Duttaphrynus melanostictus* in eastern Madagascar. *Biological Invasions, 21,* 1615-1626. https://doi.org/10.1007/s10530-019-01920-2

Lima, S. L. (1995). Back to the basics of anti-predatory vigilance: the group-size effect. *Animal Behaviour, 49,* 11-20. https://doi.org/10.1016/0003-3472(95)80149-9

Lindsey, E., Mehta, M., Dhulipala, V., Oberhauser, K. & Altizer, S. (2009). Crowding and disease: effects of host density on response to infection in a butterfly–parasite interaction. *Ecological Entomology, 34,* 551-561. https://doi.org/10.1111/j.1365-2311.2009.01107.x

Llewelyn, J., Bell, K., Schwarzkopf, L., Alford, R. A. & Shine, R. (2012). Ontogenetic shifts in a prey's chemical defences influence feeding responses of a snake predator. *Oecologia, 169,* 965-973. https://doi.org/10.1007/s00442-012-2268-1

Malagon, D. A., Melara, L. A., Prosper, O. F., Lenhart, S., Carter, E. D., Fordyce, J. A., Peterson, A. C., Miller, D. L. & Gray, M. J. (2020). Host density and habitat structure influence host contact rates and *Batrachochytrium salamandrivorans* transmission. *Scientific Reports, 10,* 5584. https://doi.org/10.1038/s41598-020-62351-x

McCoy, M. W. (2007). Conspecific density determines the magnitude and character of predator-induced phenotype. *Oecologia, 153,* 871-878. https://doi.org/10.1007/s00442-007-0795-y

Moore, D. J., Halliday, D. C. T., Rowell, D. M., Robinson, A. J. & Keogh, J. S. (2009). Positive Darwinian selection results in resistance to cardioactive toxins in true toads (Anura: Bufonidae). *Biology Letters, 5,* 513-516. https://doi.org/10.1098/rsbl.2009.0281

Morand, S. & Poulin, R. (1998). Density, body mass and parasite species richness of terrestrial mammals. *Evolutionary Ecology, 12,* 717-727. https://doi.org/10.1023/A:1006537600093

Morin, P. J. (1986). Interactions between intraspecific competition and predation in an amphibian predator-prey system. *Ecology, 67,* 713-720. https://doi.org/10.2307/1937694

Núñez-Pons, L., Carbone, M., Vázquez, J., Rodríguez, J., Nieto, R. M., Varela, M. M., Gavagnin, M. & Avila, C. (2012). Natural products from Antarctic colonial ascidians of the genera *Aplidium* and *Synoicum*: Variability and defensive role. *Marine Drugs, 10,* 1741-1764. https://doi.org/10.3390/md10081741

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

Phillips, B. L. & 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. https://doi.org/10.1098/rspb.2006.3479

Pike, N. (2011). Using false discovery rates for multiple comparisons in ecology and evolution. *Methods in Ecology and Evolution, 2,* 278-282. https://doi.org/10.1111/j.2041-210X.2010.00061.x

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team (2017). nlme: Linear and nonlinear mixed effects models. *R package version 3.1-131*.

Poitrineau, K., Brown, S. P. & Hochberg, M. E. (2003). Defence against multiple enemies. *Journal of Evolutionary Biology, 16,* 1319-1327. https://doi.org/10.1046/j.1420-9101.2003.00585.x

Pulliam, H. R. (1973). On the advantages of flocking. *Journal of Theoretical Biology, 38,* 419-422.

R Development Core Team (2017). A language and environment for statistical computing. *Vienna, Austria.*

Regueira, E., Dávila, C. & Hermida, G. N. (2016). Morphological changes in skin glands during development in *Rhinella arenarum* (Anura: Bufonidae). *The Anatomical Record, 299,* 141-156. https://doi.org/doi:10.1002/ar.23284

Relyea, R. A. (2002). Competitor-induced plasticity in tadpoles: Consequences, cues, and connections to predator-induced plasticity. *Ecological Monographs, 72,* 523-540. https://doi.org/10.1890/0012-9615(2002)072[0523:CIPITC]2.0.CO;2

Relyea, R. A. (2004). Fine-tuned phenotypes: Tadpole plasticity under 16 combinations of predators and competitors. *Ecology, 85,* 172-179. https://doi.org/10.1890/03-0169

Relyea, R. A. & Auld, J. R. (2005). Predator- and competitor-induced plasticity: How changes in foraging morphology affect phenotypic trade-offs. *Ecology, 86,* 1723-1729. https://doi.org/10.1890/04-1920

Relyea, R. A. & Hoverman, J. T. (2003). The impact of larval predators and competitors on the morphology and fitness of juvenile treefrogs. *Oecologia, 134,* 596-604. https://doi.org/10.1007/s00442-002-1161-8

Roberts, G. (1996). Why individual vigilance declines as group size increases. *Animal Behaviour, 51,* 1077-1086. https://doi.org/10.1006/anbe.1996.0109

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

Sanchez, J. N. & Hudgens, B. R. (2019). Impacts of heterogeneous host densities and contact rates on pathogen transmission in the Channel Island fox (*Urocyon littoralis*). *Biological Conservation, 236,* 593-603. https://doi.org/10.1016/j.biocon.2019.05.045

Schierling, A., Seifert, K., Sinterhauf, S. R., Rieß, J. B., Rupprecht, J. C. & Dettner, K. (2013). The multifunctional pygidial gland secretion of the Steninae (Coleoptera: Staphylinidae): ecological significance and evolution. *Chemoecology, 23,* 45-57. https://doi.org/10.1007/s00049-012-0118-6

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

Shine, R. (2010). The ecological impact of invasive cane toads (*Bufo marinus*) in Australia. *The Quarterly Review of Biology, 85,* 253-291. https://doi.org/10.1086/655116

Sih, A., Englund, G. & Wooster, D. (1998). Emergent impacts of multiple predators on prey. *Trends in Ecology & Evolution, 13,* 350-355. https://doi.org/10.1016/S0169-5347(98)01437-2

Skelly, D. K. & Werner, E. E. (1990). Behavioral and life-historical responses of larval american toads to an odonate predator. *Ecology, 71,* 2313-2322. https://doi.org/10.2307/1938642

Smith, M. J., Telfer, S., Kallio, E. R., Burthe, S., Cook, A. R., Lambin, X. & Begon, M. (2009). Host–pathogen time series data in wildlife support a transmission function between density and frequency dependence. *Proceedings of the National Academy of Sciences, 106,* 7905-7909. https://doi.org/10.1073/pnas.0809145106

Somaweera, R., Webb, J. k., Brown, G. P. & Shine, R. (2011). Hatchling Australian freshwater crocodiles rapidly learn to avoid toxic invasive cane toads. *Behaviour, 148,* 501. https://doi.org/10.1163/000579511X565763

Tempone, A. G., Pimenta, D. C., Lebrun, I., Sartorelli, P., Taniwaki, N. N., de Andrade Jr, H. F., Antoniazzi, M. M. & Jared, C. (2008). Antileishmanial and antitrypanosomal

activity of bufadienolides isolated from the toad *Rhinella jimi* parotoid macrogland secretion. *Toxicon, 52,* 13-21. https://doi.org/10.1016/j.toxicon.2008.05.008

Teplitsky, C., Plenet, S. & Joly, P. (2004). Hierarchical responses of tadpoles to multiple predators. *Ecology, 85,* 2888-2894. https://doi.org/10.1890/03-3043

Thoms, C. & Schupp, P. J. (2007). Chemical defense strategies in sponges: a review. In M. R. Custódio, G. Lôbo-Hajdu, E. Hajdu & G. Muricy (Eds.), *Porifera research: biodiversity, innovation and sustainability* (pp. 627-637). Museu Nacional.

Toledo, R. C. & Jared, C. (1995). Cutaneous granular glands and amphibian venoms. *Comparative Biochemistry and Physiology A: Physiology, 111,* 1-29. https://doi.org/10.1016/0300-9629(95)98515-I

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

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

Üveges, B., Basson, A. C., Móricz, Á. M., Bókony, V. & Hettyey, A. (2021). Dataset: Chemical defence against multiple enemies: does the response to conspecifics alleviate the response to predators? *figshare,* http://dx.doi.org/10.6084/m9.figshare.11353637

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

Üveges, B., Szederkényi, M., Mahr, K., Móricz, Á. M., Krüzselyi, D., Bókony, V., Hoi, H. & Hettyey, A. (2019). Chemical defense of toad tadpoles under risk by four predator species. *Ecology and Evolution, 9,* 6287-6299. https://doi.org/10.1002/ece3.5202

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. https://doi.org/10.1086/340599

Van Buskirk, J. (2009). Natural variation in morphology of larval amphibians: Phenotypic plasticity in nature? *Ecological Monographs, 79,* 681-705. https://doi.org/10.1890/08-1692.1

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

von Frisch, K. (1942). Über einen Schreckstoff der Fischhaut und seine biologische Bedeutung. *Zeitschrift für vergleichende Physiologie, 29,* 46-145. https://doi.org/10.1007/BF00304445

Watt, P. J., Nottingham, S. F. & Young, S. (1997). Toad tadpole aggregation behaviour: evidence for a predator avoidance function. *Animal Behaviour, 54,* 865-872. https://doi.org/10.1006/anbe.1996.0512

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

Werner, E. E. & Anholt, B. R. (1993). Ecological consequences of the trade-off between growth and mortality rates mediated by foraging activity. *The American Naturalist, 142,* 242-272. https://doi.org/10.1086/285537

West-Eberhard, M. J. (2003). Developmental Plasticity and Evolution. Oxford University Press.

Wiackowski, K. & Starońska, A. (1999). The effect of predator and prey density on the induced defence of a ciliate. *Functional Ecology, 13,* 59-65. https://doi.org/10.1046/j.1365-2435.1999.00282.x

Wildy, E. L., Chivers, D. P., Kiesecker, J. M. & Blaustein, A. R. (2001). The effects of food level and conspecific density on biting and cannibalism in larval long-toed salamanders, *Ambystoma macrodactylum. Oecologia, 128,* 202-209. https://doi.org/10.1007/s004420100641

Table 1. The effect of conspecific density and predator treatments and their interaction on chemical defence of toad tadpoles, shown as type-2 analysis-of-deviance tables. Abbreviations: TBQ: total bufadienolide quantity, mcTBQ: mass-corrected total bufadienolide quantity, NBC: number of bufadienolide compounds. Significant terms are highlighted in bold.

Response	Effect	χ^2	df	*P*
TBQ	**conspecific density**	**7.840**	**2**	**0.020**
	predator treatment	3.590	1	0.058
	conspecific density × predator treatment	0.124	2	0.940
mcTBQ	**conspecific density**	**66.551**	**2**	**<0.001**
	predator treatment	**23.801**	**1**	**<0.001**
	conspecific density × predator treatment	0.400	2	0.819
NBC	conspecific density	3.269	2	0.195
	predator treatment	0.000	1	1.000
	conspecific density × predator treatment	2.373	2	0.305

Fig. 1. Schematic illustration of density-dependent adjustments of inducible defences. Both defensive traits (A and B) provide anti-predatory protection, and their expression level increases in response to increased predation risk. For simplicity, we assume a linear (type 1) functional response of predators to prey density. For an anti-predatory defence that is disadvantageous in competition (A), expression level decreases in response to increased conspecific density due decreased pay-off. For a defence which is effective against both predators and conspecifics (B), expression level is increased by higher conspecific density due to its benefits against competitors and infections, diminishing the need for further increases of expression in response to predation risk.
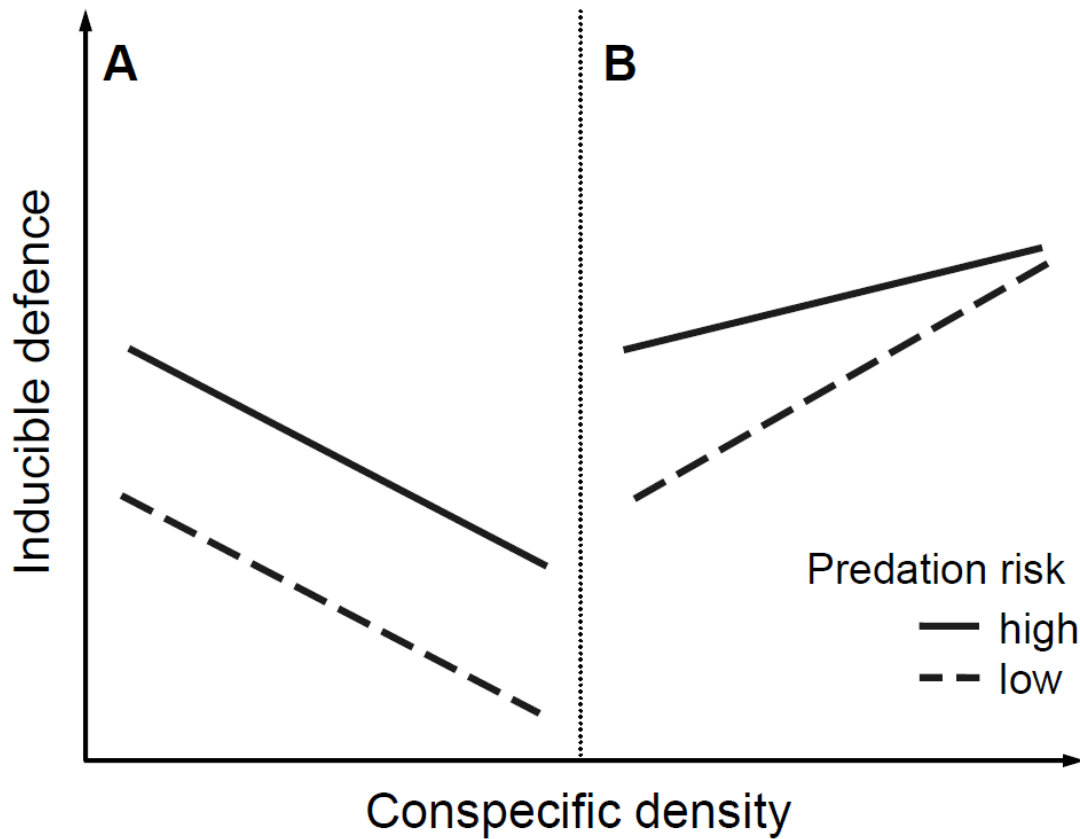
Fig. 2. Effects of predation risk and number of conspecifics on chemical defence of toad tadpoles. For total bufadienolide quantity (TBQ) and mass-corrected total bufadienolide quantity (mcTBQ), means ± SE are shown (panels A-B). Significant differences between groups, based on linear contrasts corrected for false discovery rate, are indicated by lower case letters (between density treatments; groups indicated by different plain letters differ significantly at $P < 0.05$, whereas a marginally non-significant difference ($P = 0.064$) is italicized) and asterisks (between predator treatments within each density treatment; $^{**}P < 0.01$). For the number of bufadienolide compounds (NBC), boxplots are shown (panel C), and differences between groups are not indicated because all were non-significant ($P > 0.05$). In each boxplot, the thick horizontal line and the box represent the median and the interquartile range, respectively; whiskers extend to the upper and lower quartile ± 1.5 × interquartile range, and open circles represent extreme data points.
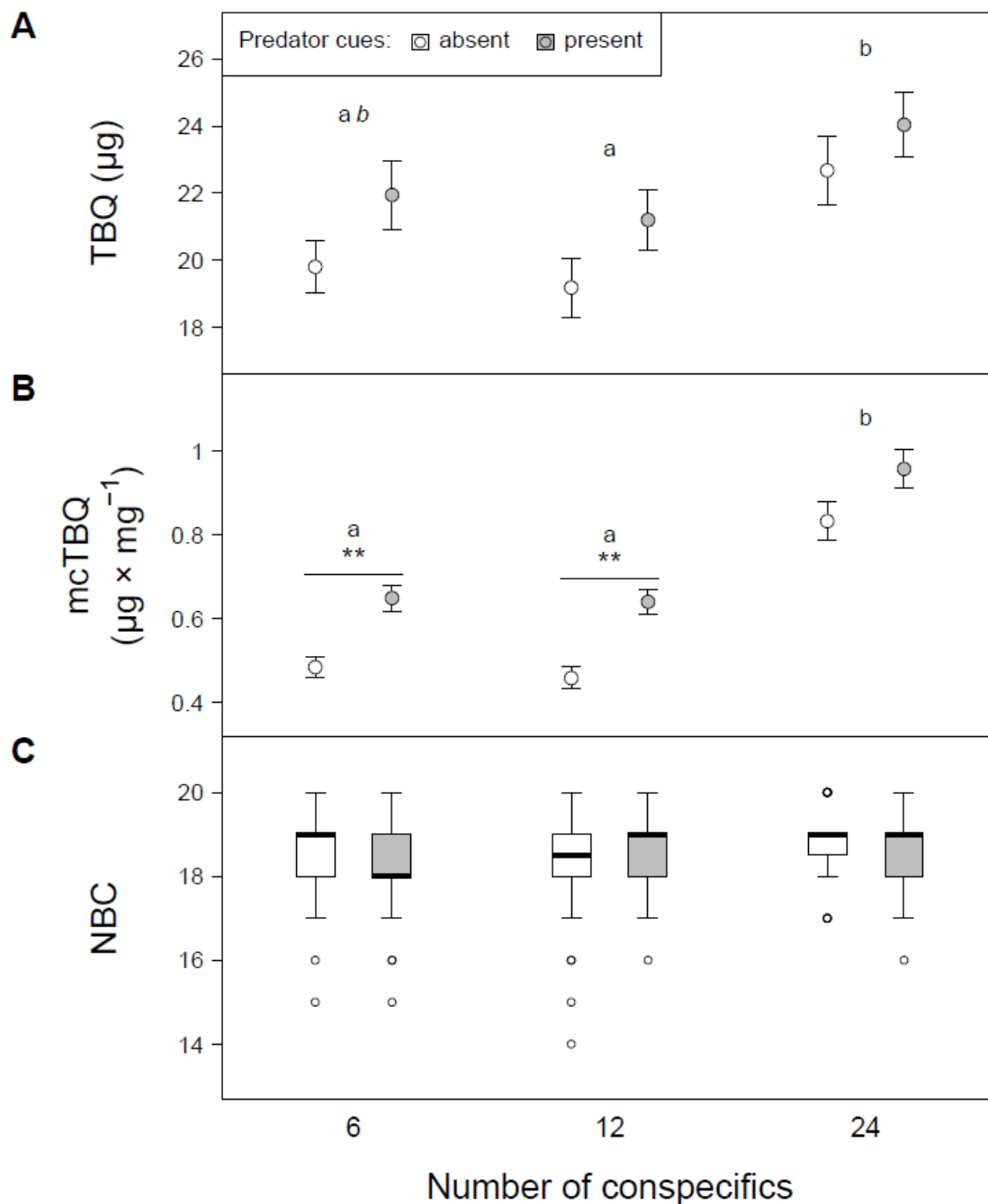
Chemical defence effective against multiple enemies: does the response to conspecifics alleviate the response to predators?

Bálint Üveges, Anna C. Basson, Ágnes M. Móricz, Veronika Bókony, Attila Hettyey

Contents

Table S1. The effect of conspecific density and predator treatments and their interaction on dry

mass of toad tadpoles, shown as type-2 analysis-of-deviance tables.

Dependent variable	Effect	χ^2	df	P
Dry mass	conspecific density	136.546	2	<0.001
	predator treatment	45.996	1	<0.001
	conspecific density × predator treatment	12.730	2	0.002

Table S2. Pairwise comparisons of the effect of conspecific density on chemical defence and dry mass of toad tadpoles, averaged for treatment groups with and without cues indicating predation risk, and within each predation treatment group, calculated using linear contrasts corrected for false discovery rate. Abbreviations: TBQ: total bufadienolide quantity, mcTBQ: mass-corrected total bufadienolide quantity, NBC: number of bufadienolide compounds; degrees of freedom = 42 (n = 288 tadpoles, 48 mesocosms).

Response	Predation risk	Contrast	Estimate	SE	t	*P*
TBQ (µg)	Averaged	6 vs 12	-0.681	1.189	-0.572	0.570
		6 vs 24	2.482	1.189	2.087	0.064
		12 vs 24	3.163	1.189	2.660	0.033
	Control	6 vs 12	-0.618	1.682	-0.368	0.715
		6 vs 24	2.873	1.682	1.708	0.143
		12 vs 24	3.491	1.682	2.076	0.132
	Fish	6 vs 12	-0.743	1.682	-0.442	0.661
		6 vs 24	2.092	1.682	1.244	0.331
		12 vs 24	2.835	1.682	1.686	0.298
mcTBQ (µg×mg^{-1})	Averaged	6 vs 12	-0.017	0.038	-0.464	0.645
		6 vs 24	0.328	0.046	7.193	<0.001
		12 vs 24	0.345	0.045	7.608	<0.001
	Control	6 vs 12	-0.026	0.049	-0.530	0.599
		6 vs 24	0.348	0.063	5.554	<0.001
		12 vs 24	0.378	0.063	5.937	<0.001
	Fish	6 vs 12	-0.009	0.057	-0.155	0.878
		6 vs 24	0.308	0.066	4.647	<0.001
		12 vs 24	0.317	0.065	4.845	<0.001
NBC	Averaged	6 vs 12	-0.115	0.213	-0.539	0.593
		6 vs 24	0.260	0.213	1.225	0.341
		12 vs 24	0.375	0.213	1.764	0.255
	Control	6 vs 12	-0.438	0.301	-1.455	0.230
		6 vs 24	0.146	0.301	0.485	0.630
		12 vs 24	0.583	0.301	1.941	0.177
	Fish	6 vs 12	0.208	0.301	0.693	0.582
		6 vs 24	0.375	0.301	1.248	0.582
		12 vs 24	0.167	0.301	0.554	0.582

Dry mass (mg)	Averaged	6 vs 12	0.545	1.180	0.461	0.647
		6 vs 24	-10.944	1.080	-10.124	<0.001
		12 vs 24	-11.489	1.130	-10.169	<0.001
	Control	6 vs 12	1.619	1.840	0.879	0.384
		6 vs 24	-13.669	1.570	-8.717	<0.001
		12 vs 24	-15.287	1.760	-8.689	<0.001
	Fish	6 vs 12	-0.529	1.480	-0.357	0.723
		6 vs 24	-8.219	1.490	-5.522	<0.001
		12 vs 24	-7.690	1.420	-5.424	<0.001

Table S3. Effect of predation risk on responses of *Bufo bufo* tadpoles (i.e. difference between groups with and without cues indicating predation risk) at each conspecific density, calculated using linear contrasts corrected for false discovery rate. Abbreviations: TBQ: total bufadienolide quantity, mcTBQ: mass-corrected total bufadienolide quantity, NBC: number of bufadienolide compounds; degrees of freedom = 42 (n = 288 tadpoles, 48 mesocosms).

Response	Conspecific density	Estimate	SE	t	*P*
TBQ (µg)	6	2.142	1.682	1.273	0.356
	12	2.017	1.682	1.199	0.356
	24	1.361	1.682	0.809	0.423
mcTBQ (µg×mg^{-1})	6	0.165	0.053	3.080	0.006
	12	0.182	0.053	3.445	0.004
	24	0.125	0.074	1.687	0.099
NBC	6	-0.292	0.301	-0.970	0.506
	12	0.354	0.301	1.178	0.506
	24	-0.063	0.301	-0.208	0.836
Dry mass (mg)	6	-7.770	1.610	-4.841	<0.001
	12	-9.920	1.730	-5.720	<0.001
	24	-2.320	1.450	-1.604	0.116

Table S4. Pairwise comparisons of the responses of toad tadpoles to the presence of cues indicating predation risk between conspecific densities, calculated as linear contrasts of the within-density contrasts (Table S2) corrected for false discovery rate. Abbreviations: TBQ: total bufadienolide quantity, mcTBQ: mass-corrected total bufadienolide quantity, NBC: number of bufadienolide compounds; degrees of freedom = 42 (n = 288 tadpoles, 48 mesocosms).

Response	Contrast	Estimate	SE	t	*P*
TBQ (μg)	6 vs 12	0.125	2.378	0.052	0.958
	6 vs 24	0.780	2.378	0.328	0.958
	12 vs 24	0.656	2.378	0.276	0.958
	6 & 12 vs 24	0.718	2.060	0.349	0.958
mcTBQ (μg×mg^{-1})	6 vs 12	-0.017	0.075	-0.229	0.820
	6 vs 24	0.040	0.091	0.440	0.820
	12 vs 24	0.057	0.091	0.631	0.820
	6 & 12 vs 24	0.049	0.083	0.588	0.820
NBC	6 vs 12	-0.646	0.425	-1.519	0.545
	6 vs 24	-0.229	0.425	-0.539	0.790
	12 vs 24	0.417	0.425	0.980	0.665
	6 & 12 vs 24	0.094	0.368	0.255	0.800
Dry mass (mg)	6 vs 12	2.150	2.360	0.909	0.369
	6 vs 24	-5.450	2.160	-2.521	0.021
	12 vs 24	-7.600	2.260	-3.363	0.003
	6 & 12 vs 24	-6.520	1.870	-3.490	0.003

Table S5. The effects of conspecific density, predator treatments, z-transformed dry mass and their interactions on total bufadienolide quantity of toad tadpoles, shown as type-2 analysis-of-deviance tables. Abbreviations: TBQ: total bufadienolide quantity.

Response	Effect	χ^2	df	P
TBQ	**density**	**7.862**	**2**	**0.020**
	dry mass	0.040	1	0.841
	predator treatment	3.601	1	0.058
	density × dry mass	1.999	2	0.368
	density × predator treatment	0.125	2	0.940
	dry mass × predator treatment	0.068	1	0.795
	density × dry mass × predator treatment	1.493	2	0.474

Fig S1. Effects of predation risk and number of conspecifics on dry mass of toad tadpoles (means ± SE). Significant differences between groups, based on linear contrasts corrected for false discovery rate, are indicated by lower case letters (between density treatments; groups indicated by different letters differ significantly at $P < 0.05$) and asterisks (between predator treatments within each density treatment; $^{***}P < 0.001$).
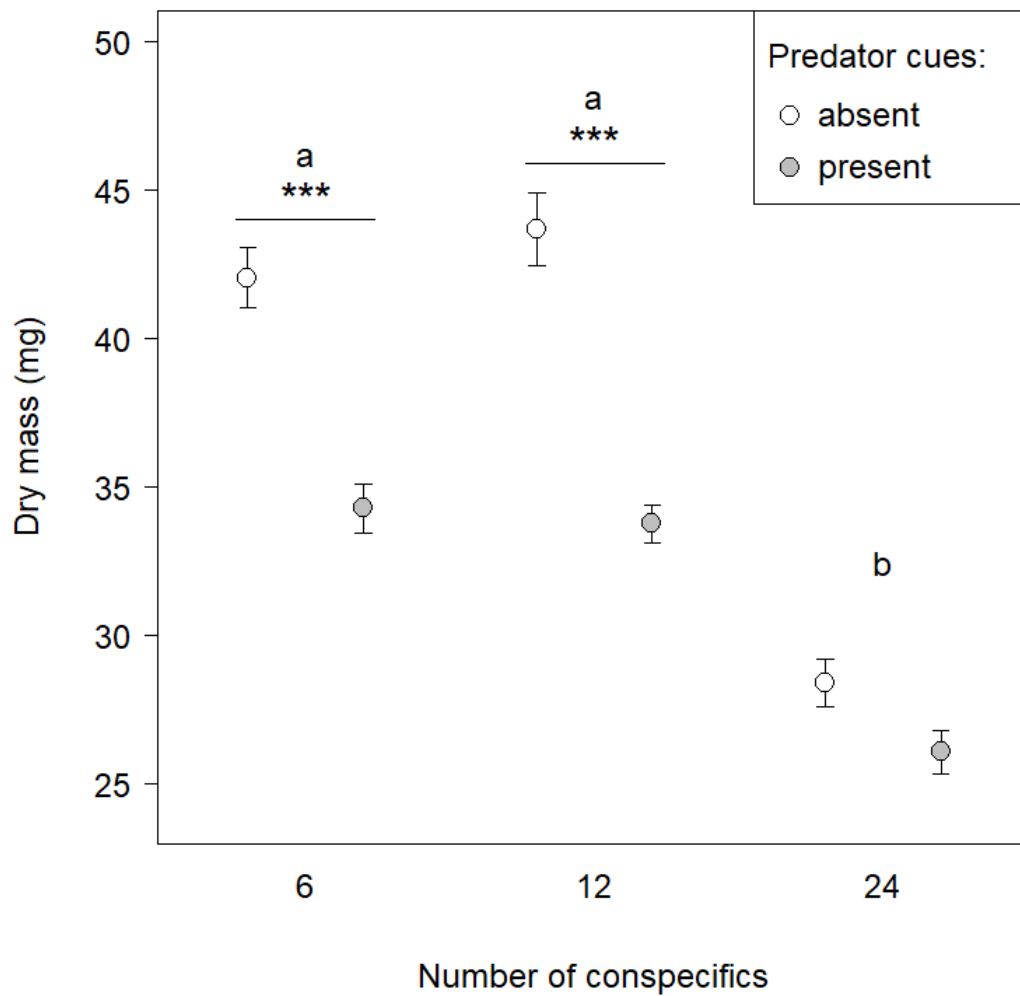
Fig. S2. Means ± 95 and 84% confidence intervals (CI) of the responses of toad tadpoles to predation risk (i.e. difference between groups with and without cues indicating predation risk) at each conspecific density. In case of each response variable, the 95% CIs that do not overlap with 0 (marked by horizontal dotted line) indicate a significant effect of predation risk, whereas 84% CIs that do not overlap with each other indicate significant differences in the intensity of responses induced by cues indicating predation risk between density treatments (Payton et al., 2003). Abbreviations: TBQ: total bufadienolide quantity, mcTBQ: mass-corrected total bufadienolide quantity, NBC: number of bufadienolide compounds.
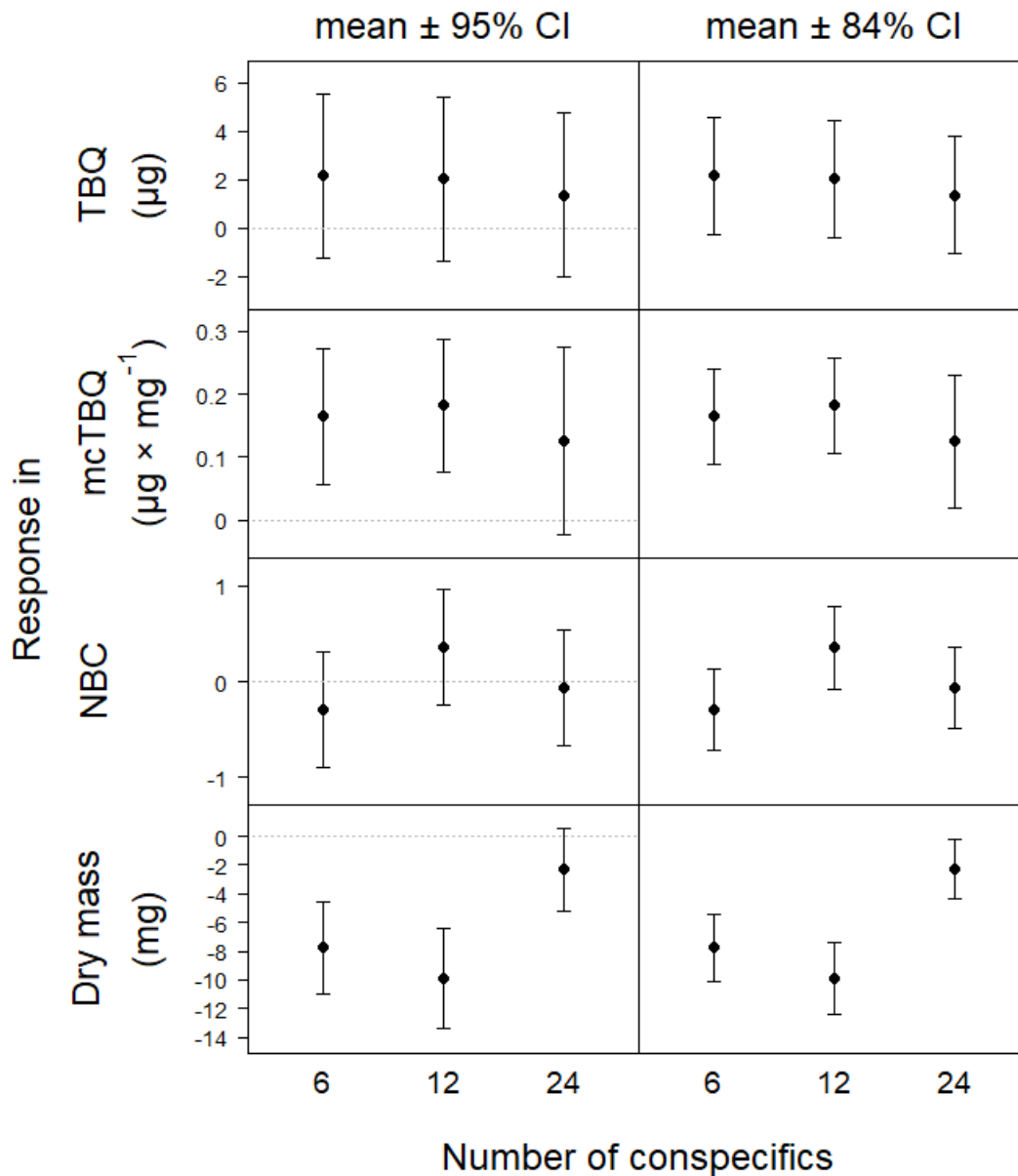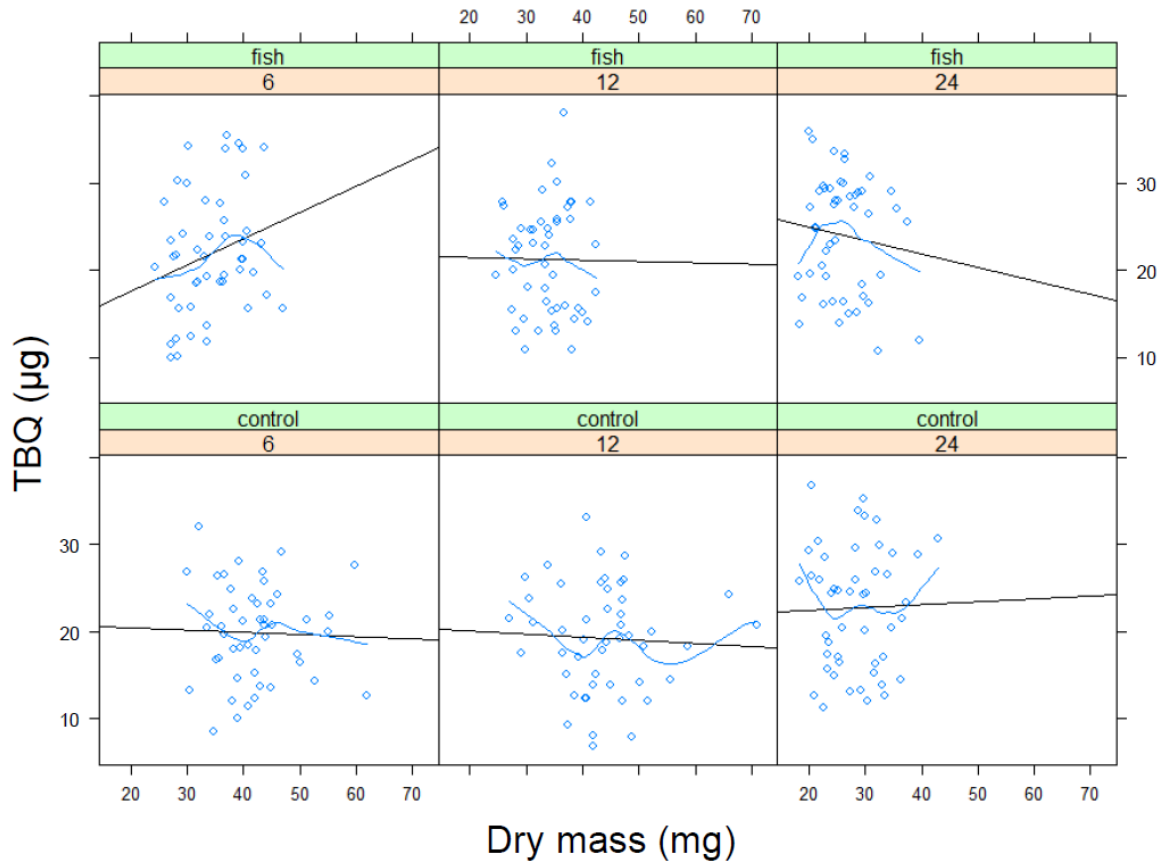
Fig. S3. Relationship between total bufadienolide quantity (TBQ) and dry mass (mg) in the treatment groups of the current study. Predator treatments: fish and control, density treatments: 6, 12 and 24.

Annotated R script

```
#R scripts in blue, comments in black.
```
#1: Reading data and setting factors
```
dataTox=read.csv("Predation Competition Toad toxins_data.csv",
header=T, sep=";")
dataTox$ftub=as.factor(dataTox$tub) #tub = mesocosm identity
dataTox$fdens=as.factor(dataTox$dens) #dens = conspecific density
dataTox$fdens=factor(dataTox$fdens, levels=c("24","12","6"))
```

#2: Loading packages
```
library(nlme)
library(emmeans)
```

#3: LMM models:
#3.1: Total bufadienolide quantity (TBQ, ng)
```
m1<-lme(TBQ~pred*fdens, random=~1|ftub, data=dataTox) #pred = predator
```
treatment

#3.2: Mass-corrected total bufadienolide quantity (mcTBQ, ng/mg)
```
m2<-lme(mcTBQ~pred*fdens, random=~1|ftub,
weights=varIdent(form=~1|pred*fdens), data=dataTox)
```

#3.3: Number of bufadienolide compounds (NBC)
```
m3<-lme(NBC~pred*fdens, random=~1|ftub, data=dataTox)
```

#3.4: Dry mass (massmg, mg)
```
m4<-lme(massmg~pred*fdens, random=~1|ftub,
weights=varIdent(form=~1|pred*fdens), data=dataTox)
```


#4: Linear contrasts. Repeat same process for each LMM model:
#4.1: Contrasts estimating the effect of conspecific density (regardless of predator treatment), for Table S2:
```
e0<-emmeans(m1, pairwise~fdens,adjust="fdr")
```

#4.1.1: Displaying results
```
summary(e0)
```

#4.2: Contrasts estimating the effect of conspecific density within predator treatments, for Table S2:
```
e0.1<-emmeans(m1, pairwise~fdens|pred,adjust="fdr")
```

#4.2.1: Displaying results:
```
summary(e0.1)
```


#4.3: Contrasts estimating the effect of predator treatment within each conspecific density, for Table S3:
```
e1<-emmeans(m1, pairwise~pred|fdens)
```

#4.3.1: Displaying results:
```
summary(e1)
```

#4.3.2: Adjusting *P* values for false discovery rate:
```
(e2=update(e1$contrasts, by = NULL, adjust="fdr"))
```

#4.3.3: Listing statistics (estimated means, SE, df, lower and upper confidence limit) for each treatment group:
```
(e3=update(e1$emmeans, by = NULL, adjust="none"))
```

#4.3.4: Saving the contrasts of predator effects within each conspecific density for further calculation:
```
fish.effects=contrast(e3,
                      list(fish6=c(-1,1, 0,0, 0,0),
                           fish12=c(0,0, -1,1, 0,0),
                           fish24=c(0,0, 0,0, -1,1)))
```

#4.4: Estimating the differences in the effect of predator treatment between conspecific densities, for Table S4:
```
dens.on.fisheff=contrast(fish.effects,
                         list(d6_12=c(1,-1,0),
                              d6_24=c(1,0,-1),
                              d12_24=c(0,1,-1)))
```

#Displaying results:
```
dens.on.fisheff
```

#4.5: Comparing the effect of predator treatment between the two lower versus the highest conspecific density for Table S4:
```
dens.on.fisheff.24.vs.6_12=contrast(fish.effects,
list(d=c(0.5,0.5,-1))))
```

#Displaying results:
```
dens.on.fisheff.24.vs.6_12
```

#Adjusting *P* values of "dens.on.fisheff" and "dens.on.fisheff.24.vs.6_12" for false discovery rate:
```
p.adjust(c(p1,p2,p3,p4), method="fdr") # p1-4 = P values of the previous
```
contrasts

References

Payton, M.E., Greenstone, M.H. & 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. https://doi.org/10.1093/jis/3.1.34