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

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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 also be 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; Úveges 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 NaHCO₃, 30 mg×L⁻¹ CaSO₄ × 2 H₂O, 61 mg×L⁻¹ MgSO₄ × 7 H₂O, 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; Schoepfner & 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), digiotoxin (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 C18 guard column (4 mm x 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 N₂ gas flow: 15 L×min⁻¹; nebulizer N₂ 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.
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; Úveges 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. Úveges 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 Úveges 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, 1993; 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 (Blennnerhassett 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


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.

<table>
<thead>
<tr>
<th>Response</th>
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<th>df</th>
<th>P</th>
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<tr>
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</table>
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

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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.

<table>
<thead>
<tr>
<th>Dependent variable</th>
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**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).

<table>
<thead>
<tr>
<th>Response</th>
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<th>Contrast</th>
<th>Estimate</th>
<th>SE</th>
<th>t</th>
<th>P</th>
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### Dry mass (mg)

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<td>6 vs 24</td>
<td>12 vs 24</td>
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**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).

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## 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).

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

<table>
<thead>
<tr>
<th>Response</th>
<th>Effect</th>
<th>$\chi^2$</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBQ</td>
<td>density</td>
<td>7.862</td>
<td>2</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>dry mass</td>
<td>0.040</td>
<td>1</td>
<td>0.841</td>
</tr>
<tr>
<td></td>
<td>predator treatment</td>
<td>3.601</td>
<td>1</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td>density × dry mass</td>
<td>1.999</td>
<td>2</td>
<td>0.368</td>
</tr>
<tr>
<td></td>
<td>density × predator treatment</td>
<td>0.125</td>
<td>2</td>
<td>0.940</td>
</tr>
<tr>
<td></td>
<td>dry mass × predator treatment</td>
<td>0.068</td>
<td>1</td>
<td>0.795</td>
</tr>
<tr>
<td></td>
<td>density × dry mass × predator treatment</td>
<td>1.493</td>
<td>2</td>
<td>0.474</td>
</tr>
</tbody>
</table>
**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")`  
\# \( p_{1-4} \) = \( P \) values of the previous contrasts
References