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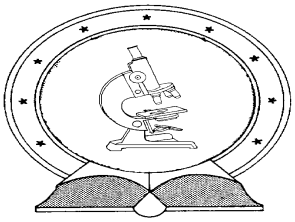

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Environmental factors shaping the variability of amphibian chemical defences

A környezet hatása kétéltűek kémiai védekezésének változatosságára

Egyetemi doktori (PhD) értekezés

Üveges Bálint

témavezető: Dr. Hettyey Attila

DEBRECENI EGYETEM

Természettudományi Doktori Tanács

Juhász-Nagy Pál Doktori Iskola

Debrecen, 2018.

Ezen értekezést a Debreceni Egyetem Természettudományi Doktori Tanács, Juhász-Nagy Pál Doktori Iskola, Biodiverzitás doktori programja keretében készítettem a Debreceni Egyetem természettudományi doktori (PhD) fokozatának elnyerése céljából. Nyilatkozom arról, hogy a tézisekben leírt eredmények nem képezik más PhD disszertáció részét.

Debrecen, 2018. július 4.

Üveges Bálint

Tanúsítom, hogy Üveges Bálint doktorjelölt 2012-2018 között a fent megnevezett Doktori Iskola Biodiverzitás programjának keretében irányításommal végezte munkáját. Az értekezésben foglalt eredményekhez a jelölt önálló alkotó tevékenységével meghatározóan hozzájárult. Nyilatkozom továbbá arról, hogy a tézisekben leírt eredmények nem képezik más PhD disszertáció részét. Az értekezés elfogadását javasolom.

Budapest, 2018. július 4.

Dr. Hettyey Attila

ENVIRONMENTAL FACTORS SHAPING THE VARIABILITY OF AMPHIBIAN CHEMICAL DEFENCES

A KÖRNYEZET HATÁSA KÉTÉLTŰEK KÉMIAI VÉDEKEZÉSÉNEK VÁLTOZATOSSÁGÁRA

Értekezés a doktori (Ph.D.) fokozat megszerzése érdekében
a biológia tudományágban

Írta: Üveges Bálint okleveles biológus

Készült a Debreceni Egyetem Juhász-Nagy Pál doktori iskolája
(Biodiverzitás programja) keretében

Témavezető: Dr. Hettyey Attila

A doktori szigorlati bizottság:

elnök:	Dr.	.....
tagok:	Dr.	.....
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A doktori szigorlat időpontja: 201… . …… … .

Az értekezés bírálói:

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Dr.	.....

A bírálóbizottság:

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Az értekezés védésének időpontja: 201… . …… … .

Contents

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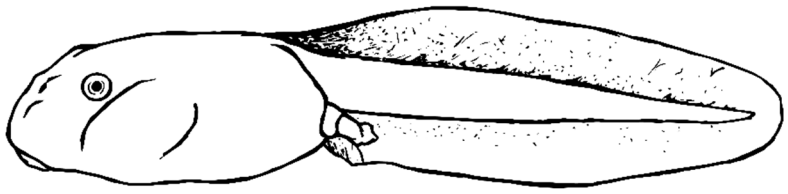
Chapter 1

Scientific background, objectives, main results and their discussion



Common toad (*Bufo bufo*) tadpole © *Viktória Verebélyi*

General introduction

Phenotypic plasticity

Populations of species constantly evolve to adapt to various environmental conditions under which they have to live and propagate. On one hand, such adaptation is achieved by gradual genetic change and subsequent natural selection of phenotypes across generations (Darwin, 1872). However, abiotic and biotic environmental conditions perpetually change even during the lifetime of individuals, therefore organisms have to possess flexible responses to be able to adapt to the diverse challenges at hand (West-Eberhard, 2003). Indeed, individuals can adjust their phenotype to environmental factors by changing their physiology, morphology, development and growth rate, as well as behaviour (Harvell, 1990; Tollrian & Harvell, 1999; West-Eberhard, 2003; Miner *et al.*, 2005). This ability of a given genotype to produce different phenotypes under different environmental conditions is called phenotypic plasticity (West-Eberhard, 1989; Futuyma, 1998; West-Eberhard, 2003; Pigliucci, 2005). Although this phenomenon is widespread in nature, it has been long shunned or even considered a problem by evolutionary biologists (Pigliucci, 2005). Recently, however, its fundamental effects on shaping diversity, and ecological and evolutionary processes have been extensively recognised (Harvell, 1990; Tollrian & Harvell, 1999; Agrawal, 2001; West-Eberhard, 2003; Miner *et al.*, 2005; Fordyce, 2006; Pfennig *et al.*, 2010). Inducible, plastic responses may be highly adaptive under variable and heterogeneous environmental conditions, however they are not without costs (DeWitt *et al.*, 1998; DeWitt & Scheiner, 2004). Furthermore, the ability to respond plastically to an inducing environmental cue can differ between habitats and populations, since local microevolutionary processes can lead to changes in the sensitivity of the genotype to environmental cues (West-Eberhard, 2003; Crispo, 2007; Pfennig *et al.*, 2010).

Inducible defences (Harvell, 1990; Tollrian & Harvell, 1999) are special cases of phenotypically plastic responses to biotic environmental factors, such as predators, competitors, pathogens and parasites, and one of their main advantages is their specificity: many organisms are capable of distinguishing between enemies and mount specific responses against them (Harvell, 1990; Crowder *et al.*, 1997; Krupa & Sih, 1998; McIntosh & Peckarsky, 1999; Tollrian & Harvell, 1999; Turner *et al.*, 1999; Relyea, 2003; Benard, 2006; Hoverman & Relyea, 2009).

An ecologically and evolutionary important adaptation in interspecific interactions is the accumulation and use of toxic substances, which is widespread in all domains of life (Keeler & Tu, 1991; Singh & Tu, 1996; Mebs, 2001; Brodie, 2009; Fry *et al.*, 2009; Yamaguchi *et al.*,

2011; Casewell *et al.*, 2013; Makarova *et al.*, 2013). While the ability of inducible expression of toxins in response to disturbance by enemies is well-known in plants (Tollrian & Harvell, 1999; Chen, 2008; McCall & Fordyce, 2010), it is scarcely studied if animals are capable of displaying plastic responses to threats by upregulating the synthesis of toxins or selectively expressing them (Hettyey *et al.*, 2014).

Inducibility of animal chemical defences

The use of toxins evolved multiple times across the animal kingdom, and is considered to be a key adaptation for predation and self-defence (Mebs, 2001; Brodie, 2009; Fry *et al.*, 2009; Casewell *et al.*, 2013). Some species acquire toxic compounds through sequestration from food or symbionts (Blum, 1981; Pawlik, 1993; Savitzky *et al.*, 2012), or obtain them from unknown sources (Tachibana, 1988; Hanifin *et al.*, 2002; Williams *et al.*, 2011), whereas others are capable of *de novo* production of toxins (Blum, 1981; Toledo & Jared, 1995; Stankowich, 2012). Apart from their function in predation (Fry *et al.*, 2009; Casewell *et al.*, 2013), the utilization of toxic or noxious compounds for deterring enemies, i.e. chemical defence, is also widespread (Mebs, 2001; Brodie, 2009). Such deterrent compounds have been identified in various taxa, and their effects on adversaries (such as predators, and potentially parasites and pathogens) are well-known in many cases (Blum, 1981; Tachibana, 1988; Pawlik, 1993; Toledo & Jared, 1995; Mebs, 2001; Cunha Filho *et al.*, 2005; Tempone *et al.*, 2008; Savitzky *et al.*, 2012). Moreover, animal toxins are intensively studied as starting points in pharmaceutical research (Fox & Serrano, 2007; King, 2011).

Given that we possess such a vast knowledge about naturally occurring toxins and their host animals, and that toxins play such an important role in the ecology and evolution of chemically defended species and their enemies (e.g. Brodie *et al.*, 2002; Hanifin *et al.*, 2008; Dobler *et al.*, 2012), it is surprising that the inducibility of chemical defences has received little attention in the past (Hettyey *et al.*, 2014). Only a few studies have tested for inducible chemical defences in animals, showing that sessile invertebrates respond to predation risk or structural damage with increased toxin production (Ebel *et al.*, 1997; Slattery *et al.*, 2001; Thornton & Kerr, 2002), and common toad (*Bufo bufo*) tadpoles respond similarly to contaminants (Bókony *et al.*, 2017). Whether predators induce toxin synthesis in vertebrate prey has remained controversial (Benard & Fordyce, 2003; Hagman *et al.*, 2009; Marion *et al.*, 2015; Bucciarelli *et al.*, 2017), since former studies tested in this regard either species (California newts, *Taricha torosa*; Bucciarelli *et al.*, 2017) whose ability to actively synthesise toxic compounds is

doubtful (Daly, 2004; Chau *et al.*, 2011; Bane *et al.*, 2014, but see Hanifin *et al.*, 2002; Lehman *et al.*, 2004), or species in which active production was not explicitly demonstrated in the same life stage when the inducing environmental factors were presented to animals (Benard & Fordyce, 2003; Hagman *et al.*, 2009; Hayes *et al.*, 2009). Furthermore, some former studies used manually simulated predator attacks (Marion *et al.*, 2015; Bucciarelli *et al.*, 2017). By using such an approach, the authors may have induced a non-selective, general stress response in their test animals rather than an anti-predator defence. Also, close to nothing is known about the effect of competitors and the costs of induced defences in this regard (Benard & Fordyce, 2003; Hagman *et al.*, 2009; Hettyey *et al.*, 2014).

Species that actively, *de novo* synthesise their toxin compounds (Chen & Osuch, 1969; Porto & Gros, 1971; Porto *et al.*, 1972) may be especially interesting to study in regard to phenotypic plasticity, because in their case the identity and quantity of compounds is largely independent of external factors and is instead to a large extent under internal control. Secondly, the inducible production of their poison is expected to come with costs, such as the maintenance of the underlying flexible synthesis pathways (DeWitt *et al.*, 1998; DeWitt & Scheiner, 2004).

Amphibians as model organisms

Amphibians are classic model organisms in phenotypic plasticity research (Miner *et al.*, 2005). Especially tadpoles of anurans (i.e. frogs and toads) have been shown to display plastic changes in morphology, behaviour and several life-history traits against various environmental factors, including predators and competitors (Laurila *et al.*, 1998; Chivers *et al.*, 1999; Lardner, 2000; Van Buskirk & McCollum, 2000; Relyea, 2001; Van Buskirk, 2002; Van Buskirk & Arioli, 2002; Relyea, 2003; 2004; Kishida & Nishimura, 2005; Steiner, 2007). Moreover, amphibians are also known to utilize a plethora of toxic compounds for self-defence (Fig. 1; Toledo & Jared, 1995; Daly, 2003), therefore they are suitable model organisms for studying the ecology and evolution of induced chemical defences.

Among anurans, bufonid toads are one of the most notorious for having toxic skin secretions (Toledo & Jared, 1995). The main toxic components of bufonid poison are bufadienolides (Fig. 1; Flier *et al.*, 1980; Krenn & Kopp, 1998; Mebs *et al.*, 2007; Gao *et al.*, 2010), which are cardiotoxic steroid derivatives with a Na^+/K^+ ATPase inhibitory function (Flier *et al.*, 1980; Pierre & Xie, 2006; Schoner & Scheiner-Bobis, 2007; Lingrel, 2010). Toads, with the exception of apparently a small number of species (Daly *et al.*, 2007; Mebs *et al.*, 2007); actively, *de novo* synthesise bufadienolides (Chen & Osuch, 1969; Porto & Gros, 1971; Porto *et al.*, 1972), and are known to contain these compounds from a very early age on (Mebs

et al., 2007; Hayes *et al.*, 2009; Bókony *et al.*, 2016; Bókony *et al.*, 2017; Ujszegi *et al.*, 2017), however, active toxin production in the tadpole stage has not been explicitly demonstrated before (Benard & Fordyce, 2003; Hayes *et al.*, 2009). Nonetheless, bufadienolides effectively deter many predator species, and even some parasites and pathogens *in vitro* (Kruse & Stone, 1984; Henrikson, 1990; Denton & Beebee, 1991; Peterson & Blaustein, 1991; Lawler & Hero, 1997; Cunha Filho *et al.*, 2005; Gunzburger & Travis, 2005; Tempone *et al.*, 2008). Furthermore, toad tadpoles have been observed to have strong negative effects on competitors,

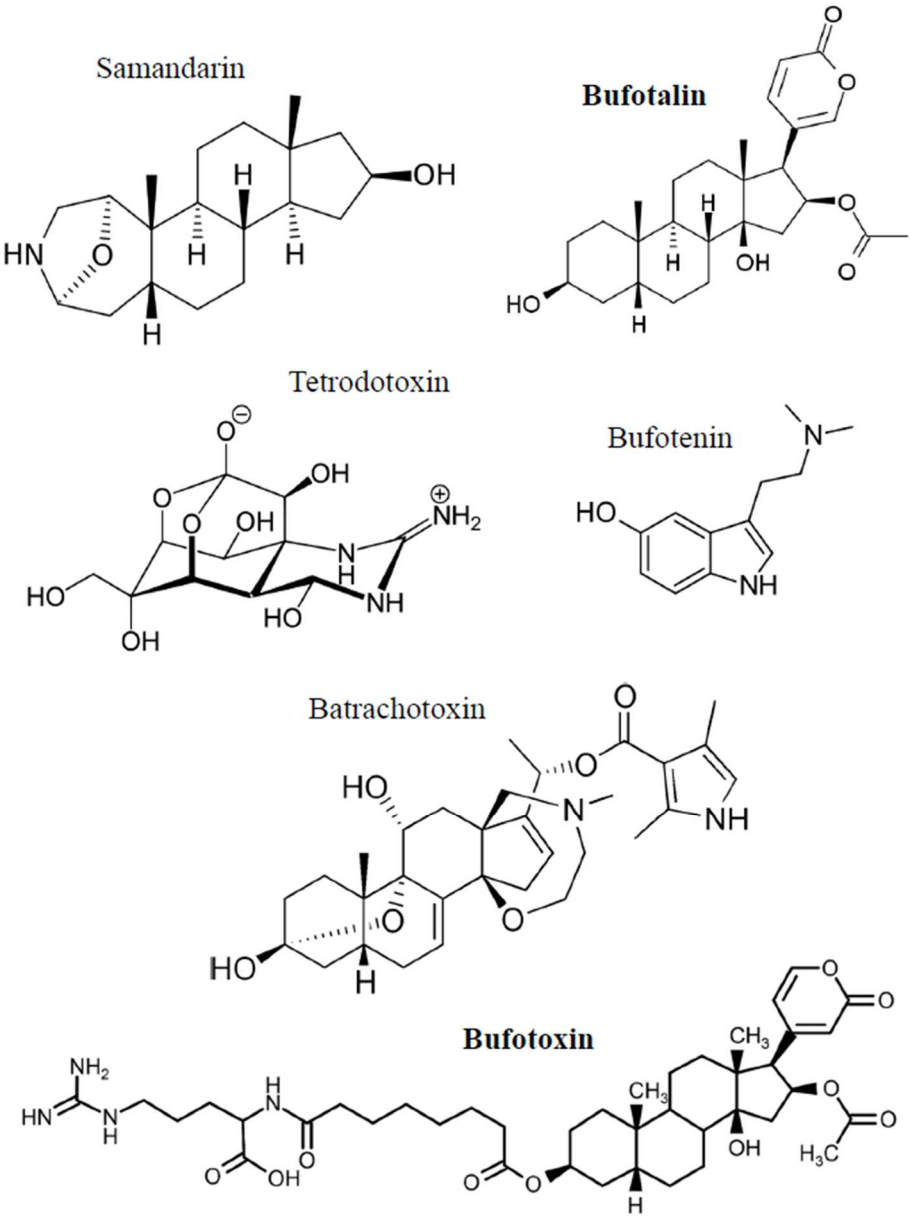


Fig. 1: Examples of amphibian toxins. Bufotalin and Bufotoxin (highlighted in bold) are characteristic bufadienolides of toads. All pictures used are in the public domain. Sources: Wikimedia Commons (https://commons.wikimedia.org) and the TOXNET Toxicology Data Network of the NIH US National Library of Medicine (Bufotoxin, https://chem.nlm.nih.gov/chemidplus/name/bufotoxin).

such as larvae of other anuran species (Licht, 1967; Wilbur & Alford, 1985). Allelopathy, intra- or interspecific competition *via* chemical compounds (Reigosa *et al.*, 2006) is a fundamentally important phenomenon in algae and plants (Metlen *et al.*, 2009; Sieg *et al.*, 2011), but its occurrence and functional importance is less understood in animals (Jackson & Buss, 1975; Kubanek *et al.*, 2002; Crossland & Shine, 2012). It has been proposed long ago, that amphibian tadpoles interfere with each other using chemical compounds (Licht, 1967), and although the mediating agents are not known, in one case (Crossland & Shine, 2012), bufadienolides have been suggested as potential culprits causing the allelopathic effect. Therefore, bufadienolides may play an important role in the defence of toads against multiple environmental threats.

Objectives and main results

In the studies presented here, our general aim was to investigate predator- and competitor-induced chemical defence in vertebrates using tadpoles of the common toad (*Bufo bufo*) as model organisms (Fig. 2). Common toad tadpoles are less plastic than other species in terms of inducible morphological or behavioural defences (Laurila *et al.*, 1998; Lardner, 2000; Van Buskirk, 2002), but contain bufadienolide toxins already in the larval stages (Mebs *et al.*, 2007; Bókony *et al.*, 2016; Bókony *et al.*, 2017; Ujszegi *et al.*, 2017) and several predators find them unpalatable (Henrikson, 1990; Denton & Beebee, 1991). This suggests that toad tadpoles are mainly dependent on an effective toxin arsenal for deterring enemies.

In our studies we tested for inducible chemical defence by manipulating exposure of common toad tadpoles to chemical cues on predation threat (Schoeppner & Relyea, 2009; Hettyey *et al.*, 2010; Hettyey *et al.*, 2015) or to competitors in laboratory and mesocosm settings. Mesocosms are self-sustaining outdoor experimental areas, which provide semi-natural circumstances for focal animals. In research involving amphibian tadpoles, mesocosms are containers in the size range from a couple to approximately 1000 litres, that contain shelter (usually leaves) and are inoculated with naturally occurring phyto- and zooplankton (e.g. Van Buskirk, 2002; Relyea, 2004; Bókony *et al.*, 2017). We also examined changes in chemical defence during ontogeny and the among population variation in inducibility of toxin production. Furthermore, we attempted to detect costs associated with induced changes in bufadienolide synthesis by manipulating the amount of food available to tadpoles. We analysed toxin content of toads using high-performance liquid chromatography with diode-array detection and mass spectrometry (HPLC-DAD-MS). For detailed information on the materials and methods used and on statistics supporting our results, please see the reprints and manuscripts in Chapter 2.

Throughout the text I refer to the corresponding scientific papers and their content using bold-faced roman numbers (**I** – **IV**).

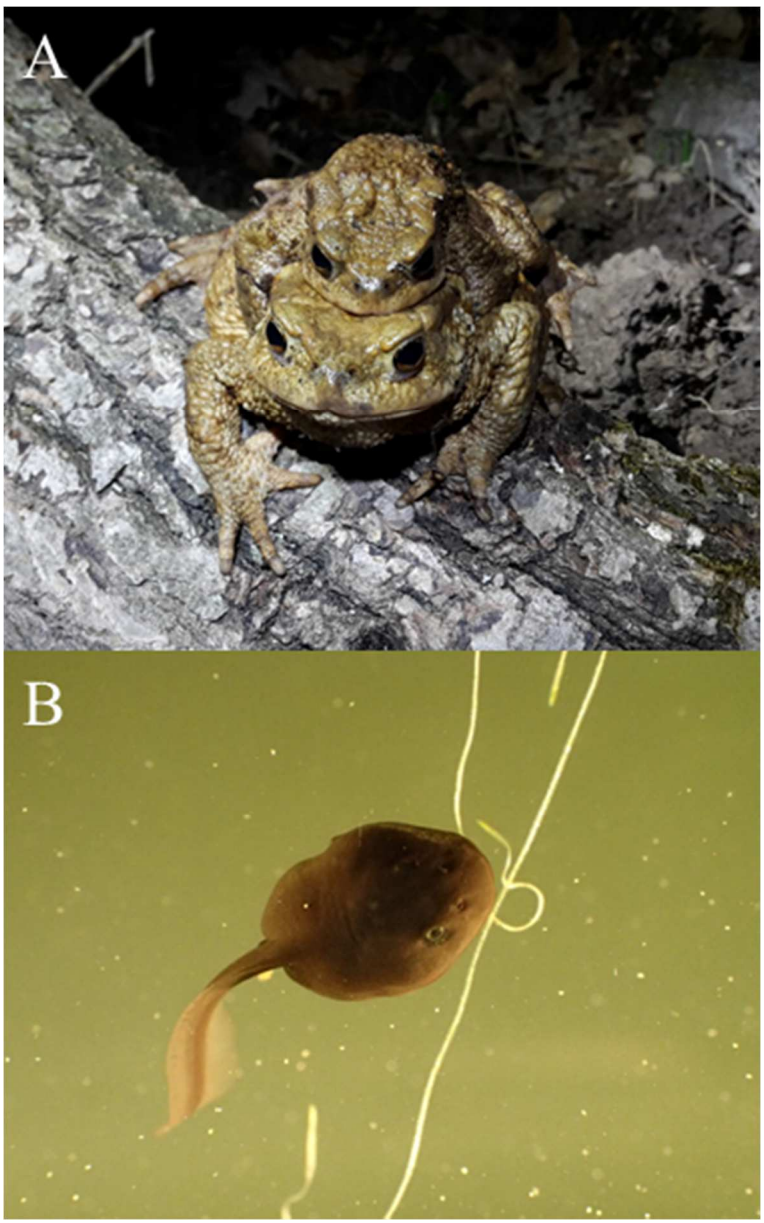


Fig. 2: A) Adult common toads (*Bufo bufo*) on their way to one of the breeding sites in the Pilis Mountains. B) A common toad tadpole feeding. Photos by the author.

Paper I *Ontogenetic variation and inducibility of chemical defence*

Aims and predictions

During this experiment, our aims were to examine ontogenetic variation in bufadienolide content of common toads during their larval and post-metamorphic life stages (Fig. 3), to investigate if cues on predation threat induce changes in toxin production during ontogeny and

to assess the energetic costs associated with plastic responses in toxin synthesis. We kept toad tadpoles in groups of three in 1.5 L water in the laboratory and exposed them to the presence or absence of chemical cues on predation risk (to manipulate the need for chemical defence) and *ad libitum* or reduced food levels (to assess the costliness of bufadienolide synthesis). The combined water of southern hawker larvae (*Aeshna cyanea*) and adult, male smooth newts (*Lissotriton vulgaris*) served as a source of predator cues. We transferred 30 ml stimulus water daily to the containers of tadpoles in the predator-cue treatment, whereas control animals received an equal amount of reconstituted soft water (RSW). We fed tadpoles with a finely grounded 4:1 mixture of rabbit chow and fish flakes. Tadpoles in the *ad libitum* treatment received a food amount of ca. 12% of their body mass/individual/day, whereas tadpoles in the reduced food treatment received one-third of that amount. Furthermore, we repeatedly sampled groups of tadpoles during development to evaluate changes in their toxin content (total N = 160).

We predicted that, if toad tadpoles are able to synthesise toxins *de novo*, they will produce higher quantities of bufadienolides when reared in the presence of cues on predation risk and will start to do so earlier in life compared to their predator-naïve conspecifics. Because *de novo* toxin synthesis is expected to come with associated energetic costs (Longson & Joss, 2006; Morgenstern & King, 2013), we also predicted that tadpoles that received reduced amounts of food would produce fewer compounds and lower quantities of toxins compared to their *ad libitum* fed conspecifics.

Main results

During their embryonal phase (developmental stage 19, Gosner, 1960) toads contained only a few bufadienolides in minute amounts (**I/**Fig. 2, **I/**Fig. 3). After hatching, tadpoles produced a significantly more diverse array of bufadienolide compounds fairly constantly during development (**I/**Table 2, **I/**Fig. 2, **I/** Table S2). Total bufadienolide quantity also significantly increased in the first half of development (to stage 34), but decreased afterwards (**I/**Table 2, **I/**Fig. 3, **I/**Table S2). Furthermore, in early life stages (developmental stages 28 and 34), tadpoles that were reared with reduced amounts of food, and consequently were of smaller size (**I/** Fig. S2), produced significantly higher quantities of bufadienolides than tadpoles that received food *ad libitum* (**I/**Table 2, **I/**Fig. 3, **I/**Table S3). On the other hand, we found no differences in the number of bufadienolide compounds or in total toxin quantity between tadpoles reared in the presence or absence of predator cues (**I/**Table 2, **I/**Fig. S4).

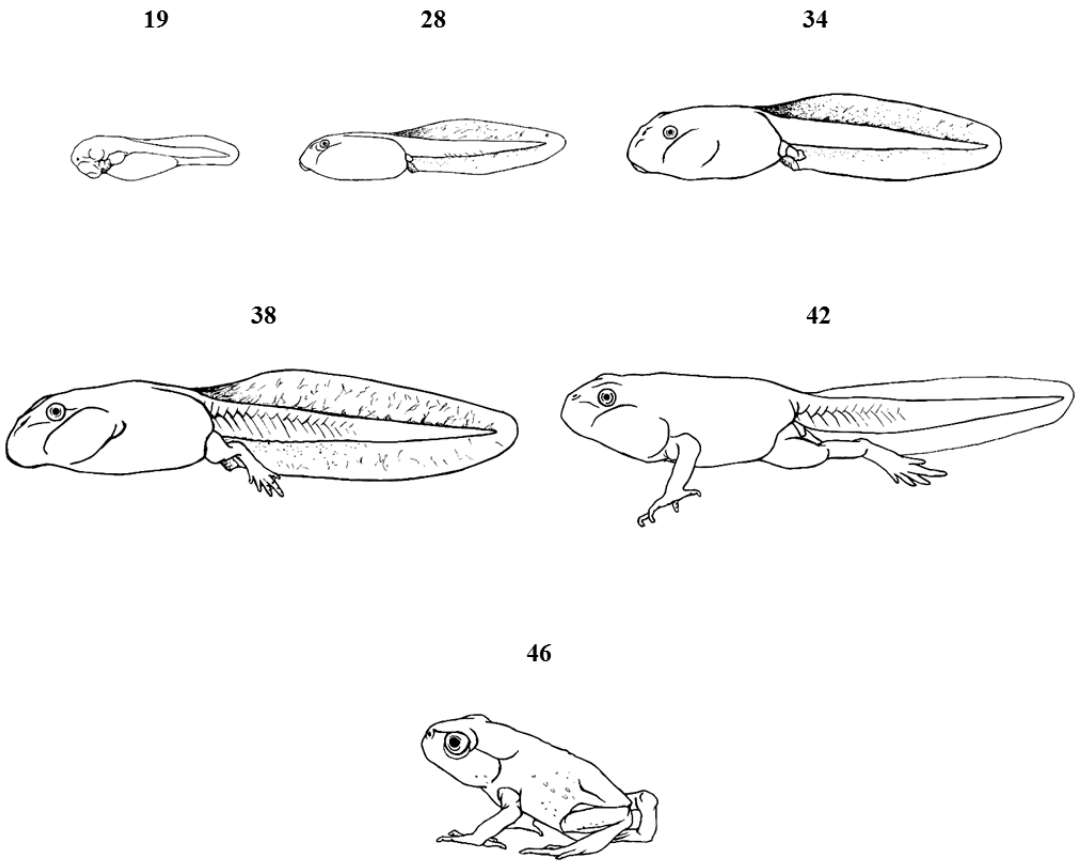


Fig. 3: Schematic representation of toad ontogeny (starting from the top left to bottom). Note, that the egg and adult phases are not presented. Numbers above figures represent developmental stages according to Gosner (1960). Drawings by Viktória Verebélyi.

Paper II *Competitor-induced chemical defence*

Aims and predictions

It is possible that reduced food levels in the previous experiment (**Paper I**) indicated high competition to toad tadpoles, inducing elevated synthesis of bufadienolide production against competitors. Additionally, one of our earlier field studies also suggested that competition may induce toxin production in common toad larvae (Bókony *et al.*, 2016). To experimentally test this hypothesis and to investigate if toad tadpoles are capable of utilizing their toxins as allelochemicals to suppress competitors, as suggested by Crossland and Shine (2012), we manipulated the density of conspecific and heterospecific tadpoles in an outdoor mesocosm experiment, using agile frog (*Rana dalmatina*) larvae as heterospecifics. We reared tadpoles in 45 L mesocosms that contained beech (*Fagus sylvatica*) leaves and were inoculated with pond-

water containing phyto- and zooplankton. We kept tadpoles in mixed family groups in the mesocosms in densities that reflected low, medium and high levels of competition based on our previous experience with the focal species. We terminated the experiment and sampled tadpoles (N = 240) after three weeks (at developmental stage 33).

We predicted that tadpoles raised under higher densities would produce a higher number of bufadienolide compounds and/or larger total bufadienolide quantity, the presence of agile frogs would have a larger effect on bufadienolide synthesis than conspecifics, and growth and development of agile frog tadpoles would be suppressed by the presence of toad tadpoles.

Main results

We found that the number of conspecifics had a marginally non-significant effect on the number of bufadienolide compounds. Bufadienolide number tended to increase with increased number of competitors, but agile frog tadpoles did not have a significant effect and treatment groups did not differ significantly from each other (**II/**Table 1, **II/**Fig. 1D). In contrast, total quantity of bufadienolides significantly increased with increasing number of conspecifics, whereas the effect of agile frog tadpoles was marginally non-significant. When present in low numbers, agile frogs tended to lead to decreased total bufadienolide quantity, and to increased toxin content of toad tadpoles as their numbers grew (**II/**Table 1, **II/**Fig. S1). The body mass-corrected quantity of bufadienolides increased gradually with competitor density and increased significantly with the number of conspecifics (**II/**Fig. 1F), whereas the number of agile frog tadpoles had no significant effect (**II/**Table 1).

The presence of toads did not exert a negative effect on frog tadpoles, since neither their body mass (**II/**Table 1, **II/**Fig. 1C), nor their developmental stage (**II/**Table 1, **II/**Fig. 2) varied significantly with the number of conspecific or heterospecific competitors.

Paper III *Inducibility of chemical defence by different predators*

Aims and predictions

In this study we investigated whether tadpoles adjust their chemical defences to predation threat in general and specifically to the presence of four, phylogenetically distantly related predator species largely differing in voraciousness, and assessed the adaptive value of the induced defence. To accomplish these goals, we reared common toad tadpoles in outdoor mesocosms in the presence or absence of caged predators (larvae of the southern hawker, *A. cyanea*, dragonfly hereafter; backswimmer imagos, *Notonecta* sp.; juvenile three-spined sticklebacks,

Gasterosteus aculeatus and adult, male smooth newts, *L. vulgaris*), measured their bufadienolide content, and finally assessed their survival upon exposure to free-ranging predators (predation trials). Mesocosms were filled with 130 L aged tap water, contained beech leaves and were inoculated with pond water. We sampled toad tadpoles on two occasions (developmental stage 29 and 42, total $N = 120$). Predation trials took place in 45 L mesocosms (similar to the ones used in **Paper I**), and were terminated when approximately half of the offered tadpoles were eaten. We collected further data on morphology, behaviour and important life-history traits of tadpoles, including body mass and length of larval development.

We predicted that tadpoles raised with caged predators would contain an elevated number of bufadienolide compounds and/or larger total bufadienolide quantity than their predator-naïve conspecifics. Also, we expected the strength of these responses to increase with predator dangerousness. Finally, we predicted that tadpoles exhibiting predator-induced phenotypes would have elevated survival probabilities compared to predator-naïve conspecifics when facing free-ranging predators.

Main results

Predator cues had no significant effect on total bufadienolide quantity (**III/**Table 2, **III/**Fig. 2, **III/**Table S1, **III/**Fig. S1) but heavier tadpoles raised in the presence of sticklebacks had fewer bufadienolides than expected from the allometric relationship between dry mass and number of compounds of control tadpoles (**III/**Table 2, **III/**Table S2, **III/**Fig. S2). This experiment corroborated our result of **Paper I**, that tadpoles (developmental stage 29) had a higher number of compounds and a higher quantity of bufadienolides compared to individuals that have started metamorphosis (developmental stage 42, **III/**Fig. 2).

We found no significant effect of predator treatment on survival, behaviour, body mass or morphology of toad tadpoles. Length of larval development was significantly shorter in the presence of sticklebacks than in control tubs (**III/**Fig. S3), whereas the other three predators did not affect this trait. I present these results in the Supplementary Information of **Paper III**.

When exposed to free-ranging dragonfly larvae, tadpoles that developed in the presence of caged specimens of this predator had significantly higher chances of survival compared to their predator-naïve conspecifics (**III/**Table 3, **III/**Fig. 3, **III/**Table S3). The presence of the other three caged predators during tadpole development did not have a significant effect on toad tadpole survival in the predation trials (**III/**Table 3, **III/**Fig. 3, **III/**Table S3).

Paper IV *Among population variation in predator-induced chemical defence*

Aims and predictions

We were unable to demonstrate predator-induced chemical defences in the aforementioned studies, but plasticity in chemical defence could have been obscured by, at least, two factors in the previous experiments, namely the presence of competitors and habitat of origin; since for both **Paper I** and **Paper III**, we collected tadpoles from a single, permanent pond and raised individuals in groups. To test for among-population differences in predator-induced chemical defences of common toads, we collected freshly laid eggs from three permanent and three temporary ponds, reared hatching larvae individually in 0.7 L RSW, either in the absence or presence of cues on predation threat in the laboratory and assessed their bufadienolide toxin content after 20 days (developmental stage 35, $N = 240$). We simulated predation threat by exposing developing tadpoles to chemical cues originating from injured conspecifics combined with the chemical cues of either larvae of the emperor dragonfly (*Anax imperator*), adult European perches (*Perca fluviatilis*) or adult, male smooth newts (*L. vulgaris*). We pipetted 20 ml freshly prepared stimulus water into rearing containers of focal tadpoles assigned to the respective predator treatments five times a week. Simultaneously, we added 20 ml RSW into rearing containers of control tadpoles.

We predicted to observe elevated bufadienolide content in tadpoles reared in the presence of cues on predation threat as compared to their predator-naïve conspecifics. We also predicted that variation in the magnitude of induced changes in toxin production would depend on the predator species. Further, we expected to find signs of local adaptation to differences in predation risk (Kawecki & Ebert, 2004) in the form of habitat-dependence of baseline toxin content (i.e. in the number and quantity of bufadienolides produced when developing in a predator-free environment) and in the intensity of antipredator responses in toxin synthesis. Continuously high predation risk imposed upon tadpoles by fishes in permanent ponds may select for more intense plastic responses in chemical defence and/or higher baseline toxin production, than weaker risk in temporary water bodies (for analogous results regarding behavioural and morphological defences see e.g. Magurran, 1990; Åbjörnsson *et al.*, 2004; Kishida *et al.*, 2007; Herczeg *et al.*, 2010; Hettyey *et al.*, 2016). On the other hand, constantly high predation risk may also reduce plasticity in defensive traits (West-Eberhard, 2003; Crispo, 2007; Pfennig *et al.*, 2010), such as toxin production. Our previous results (**Papers I** and **III**) supported the latter hypothesis, but due to the controversial evidence presented above, no clear prediction could be made regarding differences in the magnitude or direction of the inducibility of toad chemical defences between permanent and temporary waterbodies.

Main results

Tadpoles exposed to different predator treatments responded with the production of increased numbers and total quantity of bufadienolide compounds. Predator-naïve tadpoles contained significantly fewer and lower quantities of bufadienolides as compared to tadpoles exposed to cues of any type of predator (**IV**/Fig. 2, **IV**/Fig. S1). Tadpoles reared in the presence of cues from perch produced the highest quantities of bufadienolides, whereas tadpoles exposed to cues of dragonflies and newts contained intermediate toxin levels (**IV**/Table S3, **IV**/Fig. S1).

The analysis on control tadpoles reared in the absence of cues of predation threat did not reveal significant variation among populations either in the number of bufadienolide compounds or in total bufadienolide quantity. Baseline values of compound number and total bufadienolide quantity also did not differ between tadpoles originating from permanent and temporary ponds (**IV**/Fig. 1).

We detected significant variation among tadpoles according to population of origin in the intensity of predator-induced changes in the number of bufadienolide compounds (**IV**/Fig. 2). When analysing antipredator responses in compound number in tadpoles originating from the three temporary and the three permanent ponds together, we found significant changes in response to all predators in both types of water bodies, but these responses did not differ between tadpole populations originating from the two pond types (temporary vs. permanent ponds, **IV**/Table 1).

In contrast, we did not detect among-population variation in the intensity of responses in total bufadienolide quantity (**IV**/Fig. 2). When analysing antipredator responses in total bufadienolide quantity in tadpoles originating from the three temporary and the three permanent ponds together, we did not find significant changes in response to newts in either type of water body and in response to dragonflies in permanent ponds, while tadpoles originating from temporary ponds responded to dragonflies with increased toxin production, and so did tadpoles originating from both types of water bodies exposed to chemical cues of perch (**IV**/Table 1, **IV**/Fig. 2). However, we found no significant differences in the magnitude of responses between tadpoles originating from temporary and permanent ponds (**IV**/Table 1).

General discussion

Inducibility of chemical defence

Our results described in **Paper I** provide evidence for plastic changes in toxin production induced by food availability: during the first half of larval development, tadpoles that had access to only reduced amounts of food contained significantly more bufadienolides than their conspecifics that were fed *ad libitum*. Although this may seem counterintuitive, it agrees with former findings that plants may invest more resources in chemical defence when exposed to nutrient stress (Gershenzon, 1984; Fagerström *et al.*, 1987). To our knowledge, this is the first study to show such an effect in animals (Longson & Joss, 2006). As a functional cause for the plastic chemical defence demonstrated here we propose that reduced food level might have indicated high competition to tadpoles, induced increased production of bufadienolides against competitors or their pathogens and parasites. This hypothesis is supported by results of our former field study, in which we demonstrated that number of bufadienolide compounds correlated and total bufadienolide quantities tended to correlate with competitor (mainly amphibian larvae) density in natural ponds (Bókony *et al.*, 2016).

In a subsequent experiment (**Paper II**) we indeed demonstrated that toad tadpoles upregulate their chemical defence in response to an increase in density of conspecifics. This result suggests that induced chemical defences in taxa characterised by behavioural and morphological complexity may serve several functions beyond defence against predators and pathogens (Tollrian & Harvell, 1999; Hettyey *et al.*, 2014). The fact that tadpoles that were exposed to food-deprivation (**Paper I**) or more intense competition (**Paper II**) had lower body mass than their conspecifics in less stressful environments, yet were able to produce increased quantities of toxins, suggests that the energetic cost of bufadienolide synthesis in toad larvae may be low (Kurali *et al*., 2016). Ultimately, however, the cost-effectiveness of induced bufadienolide synthesis may be more important in determining its effect on fitness than the absolute energetic demand of the plastic response, but since our experiments did not test this assumption explicitly, we cannot draw definite conclusions regarding this topic. Moreover, we cannot dismiss the notion that investment in bufadienolide production may be in trade-off with other important life-history traits (Benard & Fordyce, 2003; Hagman *et al.*, 2009), but at least in the case of developmental rate, such a trade-off was not detected, since developmental stage of toad tadpoles did not differ between treatments in **Paper II**.

Toad species are known to have evolved resistance against their own bufadienolides (Moore *et al.*, 2009; Crossland *et al.*, 2011a; Crossland *et al.*, 2011b; Crossland & Shine, 2011), therefore we expected that competition from heterospecific tadpoles will be the main driver behind induced toxin synthesis. However such an effect was not apparent, since agile frog tadpoles did not have a stronger effect on the bufadienolide content of toads compared to the

same total mass of conspecifics. We would expect such a result for instance if toad tadpoles were unable to differentiate between con- and heterospecific competitors, yet this is highly unlikely (Relyea, 2002). It is more plausible, that behavioural differences between the two species caused this effect. Toad tadpoles are highly active and gregarious, whereas agile frog larvae behave more calmly and usually do not form tight groups (pers. obs.). This could have resulted in low encounter rates between individuals of the two species, which in turn would have caused a stronger effect of conspecifics if toad tadpoles assess competitor density based on physical contact or proximity (e.g. visual or tactile cues; Rot-Nikcevic *et al.*, 2005).

Alternatively, toad tadpoles may produce elevated quantities of bufadienolide toxins when reared with conspecifics as a consequence of synergistic selection (Maynard Smith, 1982; Maynard Smith, 1998; Corning & Szathmáry, 2015). In game theory, synergy describes a non-additive case of cooperation: when individuals mutually cooperate, they gain more benefits (e.g. higher fitness) than the sum of their individual scores (fitnesses) achieved by not cooperating. Through selection, such interactions then may become evolutionary stable strategies (Maynard Smith, 1982; Maynard Smith, 1998; Corning & Szathmáry, 2015). Indeed, research suggest that plant unpalatability (Tuomi & Augner, 1993; Leimar & Tuomi, 1998) and aposematic coloration of marine gastropods (Rosenberg, 1991) may evolve through synergy. Synergy requires that individuals aggregate, however group living poses a problem to animals that are small and slow compared to their predators (e.g. tadpoles compared to fish), because without some form of chemical defence predators may consume the majority or entirety of a group of such species when found. Therefore the evolution of unpalatability seems to be a crucial prerequisite for group-living in such animals (Sillén-Tullberg & Leimar, 1988; Tullberg *et al.*, 2000). In case of toads, when population density is low, tadpoles are more solitary or may even adopt a cryptic lifestyle, and consequently are less conspicuous to predators, therefore the pressure to synthesise high amounts of toxins is relaxed (Sillén-Tullberg & Leimar, 1988; Tullberg *et al.*, 2000). When density is high, however, toad tadpoles are known to form aggregations (Wells, 2007), which makes them especially easy to locate, at least for the human eye (pers. obs.) and most probably for other visually hunting predators too. In this situation elevated distastefulness is beneficial (Leimar & Tuomi, 1998) by facilitating predator avoidance learning, i.e. decreasing the number of prey sampled by predators before they are deterred from further attacks (Sillén-Tullberg & Leimar, 1988). This mechanism is further strengthened if members of the aggregation express similarly high levels of chemical defence (Rosenberg, 1991; Leimar & Tuomi, 1998). The same scenario was proposed by Sillén-Tullberg and Leimar (1988) for the evolution of gregariousness of chemically defended insects. Experimental

support for increased toxicity in animal aggregations compared to lone individuals comes from a study with the desert locust (*Schistocerca gregaria*). Solitarious-phase locusts avoid consuming alkaloid containing plants, whereas animals that have been reared with conspecifics and started the transition to the gregarious-phase morph enhance their chemical defence by preferring toxic food items (Despland & Simpson, 2005). Nonetheless, I know of no experiment to date that explicitly tested the role of synergistic selection in the origin of animal chemical defences.

Predation risk did not affect the production of bufadienolides in the first and third experiment (**Papers I** and **III**). This agreed with a former field study where we also could not detect predator effects on toxin production, since bufadienolide content of toad tadpoles did not correlate with predator density in natural ponds (Bókony *et al.*, 2016). A previous experiment (Benard & Fordyce, 2003) was also unable to find plastic changes in bufadienolide content of tadpoles in response to predator cues, but the chemical analytical method applied there was not as sensitive as in our studies and toxins were not present in the studied tadpoles in quantifiable amounts. Consequently, that study remained inconclusive in respect to predator-induced changes in toxin production during the larval stage. However, when toad tadpoles were raised with predator cues, differences in chemical defence between control and predator-exposed individuals became apparent after metamorphosis (Benard & Fordyce, 2003; Hagman *et al.*, 2009), which suggests that toads respond to larval predation risk by some physiological changes in the bufadienolide synthesis pathway or anatomical changes in toxin-producing structures that become detectable only during or after metamorphosis.

Indeed, in **Paper IV**, we present evidence for predator-induced changes in the chemical defence of common toad larvae. Tadpoles reared in the presence of chemical cues on predation threat produced a larger number of bufadienolide compounds and higher total bufadienolide quantity as compared to tadpoles that developed in a predator-free environment. Our results also provide support for the hypothesis that the intensity of induced changes in chemical defences can vary depending on the predator species present, very much like in other defensive traits (Sih, 1986; Relyea 2001; Van Buskirk & Arioli, 2002; Hettyey *et al.*, 2011). Toad tadpoles produced the highest number and quantity of bufadienolide compounds in the presence of perch, while they responded weaker to dragonfly larvae and least to newts.

How can this discrepancy with our previous studies be explained? Large differences among studies in sample sizes (60 samples per treatment in study **IV** vs. 10 and 24 replicates per treatment in the previous investigations; **Papers I** and **III**, respectively) may have resulted in differences in statistical power, contributing to our inability of detecting treatment effects in

our former studies and enhancing our capability to detect them in the latter experiment. Also, populations can vary in how plastically they respond to environmental cues (Magurran, 1990; West-Eberhard, 2003; Åbjörnsson *et al.*, 2004; Crispo, 2007; Pfennig *et al.*, 2010; Hettyey *et al.*, 2016) and in the current study (**Paper IV**), we provide evidence that this is also true for the strength of antipredator responses in toxin production. In experiments **I** and **III** we may have accidentally used populations exhibiting low levels of plasticity in chemical defences, since tadpoles in both studies originated from a permanent pond inhabited by fishes. Because fishes have persisted for many generations in these aquatic habitats, and they the most voracious predators of amphibian larvae (Semlitsch, 1993; Relyea 2001; Wells, 2007), it is possible that selection acted to reduce plasticity in bufadienolide synthesis in these populations, resulting in a constitutively expressed chemical defence (through genetic assimilation; West-Eberhard, 2003; Crispo, 2007; Pfennig *et al.*, 2010). However, former empirical evidence on morphological and behavioural plastic responses suggests that higher baseline toxin levels and /or a more intense plastic response may also evolve in such habitats (Magurran, 1990; Åbjörnsson *et al.*, 2004; Kishida *et al.*, 2007; Herczeg *et al.*, 2010; Hettyey *et al.*, 2016).

We found mixed evidence for local adaptation in chemical defence of toad tadpole populations (**Paper IV**): the magnitude of induced antipredator responses in the number of bufadienolide compounds varied significantly among the six sampled populations, while similar variation was not detectable in baseline toxin content and in the extent of induced changes in total bufadienolide quantity. The lack of systematic differences between groups of populations originating from temporary or permanent ponds was somewhat surprising, because as mentioned above, fishes are in general considered the most voracious predators of anuran larvae (Semlitsch, 1993; Relyea 2001; Wells, 2007), and failure to produce sufficiently effective defences may lead to very low survival probability in fish-infested permanent ponds. The incongruity between former empirical evidence (Magurran, 1990; Åbjörnsson *et al.*, 2004; Kishida *et al.*, 2007; Herczeg *et al.*, 2010; Hettyey *et al.*, 2016), as well as theory (West-Eberhard, 2003; Crispo, 2007; Pfennig *et al.*, 2010), and our results regarding the comparison between populations originating from temporary vs. permanent ponds may be accounted to gene flow between permanent ponds and adjacent temporary puddles obstructing local adaptation to varying levels of predation risk (Kawecki & Ebert, 2004; Yeaman & Otto, 2011; Blanquart *et al.*, 2012). Also, shallow areas inaccessible to fishes may provide suitable refugia for tadpoles in fish-infested ponds, weakening selection acting towards fixation of high levels of toxin production and of plasticity. Finally, chemical defences of toads are in general more effective against vertebrate than invertebrate predators (Henrikson, 1990; Manteifel &

Reshetnikov, 2002; Gunzburger & Travis, 2005), and already relatively low quantities of bufadienolides may provide efficient defences against fishes (see results of the predation trials in **Paper III**).

Proximate and ultimate explanations for the observed patterns in inducible chemical defence

Our result that intraspecific competitors had a greater effect on bufadienolide production than heterospecifics did (**Paper II**), suggests a function in defending against threats presented mainly by conspecific tadpoles. First, scarcity of food combined with high densities often leads to an increased occurrence of intraspecific aggressive behaviour and even cannibalism (Wildy *et al.*, 2001; Jordan *et al.*, 2004; Jefferson *et al.*, 2014; Mahapatra *et al.*, 2017). Higher toxin quantity may have the function to defend against such attacks, not by toxicity *per se*, but if toads, although resistant to bufadienolides (Moore *et al.*, 2009; Crossland *et al.*, 2011a; Crossland *et al.*, 2011b; Crossland & Shine, 2011), still find these compounds distasteful, similarly to other species (Gunzburger & Travis, 2005). On the other hand, bufadienolides may also play a role in intraspecific olfactory communication and species recognition (Hagman & Shine, 2009; Crossland & Shine, 2011), and thereby may facilitate prevention of cannibalism, especially amongst kin within schools of toad larvae (Blaustein, 1988). Bufadienolides may also defend against pathogens and parasites, and hence form a part of the immune system, since it has been demonstrated that these compounds have antimicrobial and antiparasitic effects (Cunha Filho *et al.*, 2005; Tempone *et al.*, 2008). Such a role of bufadienolides would be of great importance to toads, because they do not possess antimicrobial skin peptides, which are present in many other amphibian species (Conlon *et al.*, 2009). Our observation of increased toxin production upon exposure to high conspecific density may have been an anti-pathogenic response, because chemical defences are known to be induced by infection in amphibians (Groner *et al.*, 2014), contracting diseases is more likely at high densities (Briggs *et al.*, 2010) and individuals are more susceptible to diseases of conspecifics than those of other species (Freeland, 1983). However explicit tests of the effectiveness of induced bufadienolide synthesis in deterring cannibalism and/or prevention of contracting diseases are yet to be conducted. Of course bufadienolides may still fulfil these roles, even if elevated chemical defence in tadpole aggregations may ultimately evolve through synergistic selection to deter predators (Rosenberg, 1991; Tuomi & Augner, 1993; Leimar & Tuomi, 1998).

Whether bufadienolides play a role in interspecific chemical interference remains unclear, because the presence of toad larvae did not suppress the development and growth of

agile frog tadpoles. As mentioned above, the lack of allelopathic effects could be explained by low encounter rates between the members of the two species. The amphiphilic nature of bufadienolides means that the highest concentrations of these compounds should occur at the contact zone of toad skin and water (Kubanek *et al.*, 2002) and therefore their allelopathic effects may only materialise in case of direct physical contact, such as in situations leading to highly crowded conditions (e.g. due to desiccation of the waterbody; Cabrera-Guzmán *et al.*, 2012) or when food resources drop critically and scavenging on injured or dead tadpoles becomes more frequent (Wildy *et al.*, 2001; Jordan *et al.*, 2004; Jefferson *et al.*, 2014; Mahapatra *et al.*, 2017).

It has been long established, that one of the main functions of animal chemical defences is to deter predators (Toledo & Jared, 1995; Brodie, 2009). We found that dragonflies posed the biggest threat to toad tadpoles, followed by backswimmers, sticklebacks and newts in this order (**Paper III**). The predation trials revealed that tadpoles raised with dragonfly larvae survived better, compared to predator-naïve tadpoles, when they were exposed to this predator. Because we could not detect any significant phenotypic responses induced by the presence of caged dragonflies during tadpole development (Supplementary Information for **Paper III**), we speculate that this treatment affected some unstudied aspect of behaviour, morphology, physiology or chemical defence of tadpoles (e.g., enhanced schooling behaviour or elevated synthesis of non-bufadienolide defensive chemicals) that provided an effective defence against this predator. We did not observe differences in survival in predation trials between control tadpoles and their siblings raised with backswimmers, newts or sticklebacks, similarly to earlier findings with various predators (McCollum & Van Buskirk, 1996; Van Buskirk & Relyea, 1998). However, when confronted with these predators, especially the vertebrate species, survival of toad tadpoles was very high, leaving little variation for an effect of the rearing environment to manifest in. Similarly, during feeding sessions in the rearing stage of the experiment, caged newts and sticklebacks consumed fewer of the offered naïve toad tadpoles than did backswimmers and dragonflies. This suggests that the baseline toxin levels in the studied toad population are high enough to provide effective defence against newts and fish.

This differential susceptibility of toad tadpoles to invertebrate and vertebrate predators is consistent with earlier results: typically, invertebrates find chemically defended tadpoles more palatable than do vertebrates (Gunzburger & Travis, 2005). This difference may, at least partly, be due to disparate sensitivity to bufadienolides, which inhibit Na^+/K^+ ATPases through attaching to the oubain binding site of these enzymes (Flier *et al.*, 1980; Pierre & Xie, 2006; Schoner & Scheiner-Bobis, 2007; Lingrel, 2010). Indeed, some species find bufadienolide-

containing prey unpalatable (Kruse & Stone, 1984; Henrikson, 1990; Denton & Beebee, 1991; Peterson & Blaustein, 1991; Toledo & Jared, 1995; Lawler & Hero, 1997), while others appear to be resistant to these compounds (Dobler *et al.*, 2012; Ujvari *et al.*, 2015; Mohammadi *et al.*, 2016; Arbuckle *et al.*, 2017). The high palatability of toad tadpoles to dragonfly larvae might be due to such a resistance. Furthermore, utilizing a special feeding apparatus may also circumvent chemical defences of toad tadpoles: the pierce and suck feeding method of backswimmers may allow them to avoid the ingestion of bufadienolides produced and stored mainly in the skin of toads (Toledo & Jared, 1995; Halliday *et al.*, 2009). On the other hand, species that engulf their entire prey and do not seem to have evolved resistance against bufadienolides, such as smooth newts and sticklebacks, likely become fully exposed to the toxic effects of tadpoles' chemical defence upon ingestion.

Ontogeny of chemical defence

We found that bufadienolide quantity as well as the number of bufadienolide compounds steeply increased after hatching (**Paper I**). This indicated that tadpoles actively synthesize their toxins, which has been suggested before by studies examining the ontogenetic development of glands in the skin of common toad tadpoles (Delfino *et al.*, 1995; Chammas *et al.*, 2014). However, this result is in contrast with previous findings in other toad species (Benard & Fordyce, 2003; Hagman *et al.*, 2009; Hayes *et al.*, 2009) in which active synthesis of bufadienolides was not demonstrated during larval life, and maternal provisioning of bufadienolides or non-bufadienolide toxins were instead suspected to be of high importance in chemical defence.

After hatching, the number of bufadienolide compounds was rather constant during most of larval life (**Paper I**) and decreased by the onset of metamorphosis (**Paper III**), while total bufadienolide quantity first increased, reaching the highest levels in mid-aged larvae, and then decreased nearing metamorphosis (**Paper I** and **III**). This pattern matches the shifts occurring in vulnerability of tadpoles during development remarkably well: in the earliest life stages, amphibian larvae are susceptible to predation, therefore toxin synthesis is highly beneficial. As tadpoles grow, they may reach a size threshold, after which gape-limited predators, such as fishes or newts, may not be able to efficiently feed on them. Also enhanced swimming performance of large tadpoles makes capturing them more difficult (Semlitsch & Gibbons, 1988; Richards & Bull, 1990; Eklöv & Werner, 2000; Wilson & Franklin, 2000), therefore they may have to invest less in toxin synthesis. In general, such a link between ontogenetic changes in potential susceptibility to predators and toxin content may be common

in organisms utilizing chemical defences (Lindquist & Hay, 1996; Camarano *et al.*, 2006; Lopanik *et al.*, 2006). Furthermore, since many predators learn to avoid toxic prey (Brodie & Formanowicz, 1981; Kruse & Stone, 1984; Nelson *et al.*, 2011), tadpoles may be relieved from producing large quantities of toxic compounds later in development. Albeit metamorphosing and post-metamorphic animals had lower bufadienolide quantities than tadpoles, these amounts may still have been high enough to provide effective defence against some predator species, with the caveat that metamorphosing amphibians are more vulnerable to predation than before the start or after the completion of metamorphosis (Wassersug & Sperry, 1977; Arnold & Wassersug, 1978; Crump, 1984; Calsbeek & Kuchta, 2011; Touchon *et al.*, 2013). Alternatively, the lower amounts of bufadienolides in late larval stages may be a consequence of anatomical and energetic constraints accompanying metamorphosis, when a broad rearrangement of various organs takes place (Beck & Congdon, 2003; Brown & Cai, 2007; Orlofske & Hopkins, 2009). Other animals with complex life-histories that actively produce toxins, such as various insects (Highnam, 1981), are expected to display a similar pattern of ontogenetic variation of toxin content.

Conclusions

Together, our studies represent the most comprehensive investigation of plastic chemical defences in any animal species to date. We showed that bufadienolide synthesis of common toad tadpoles is inducible by food deprivation (**Paper I**), conspecifics (**Paper II**) and predators (**Paper IV**). Moreover we also demonstrated active synthesis of toxins in larval anurans.

It is clear from our results, that various environmental factors can induce bufadienolide synthesis in common toads (see also Bókony *et al.*, 2017), suggesting that toxins may be effective against multiple threats. Albeit we did not find allelopathic effects of these compounds against heterospecific competitors (**Paper II**), we show that the observed bufadienolide levels effectively deter vertebrate predators, but are less efficient in fending off invertebrates, which may have evolved adaptations to mitigate effects of bufadienolides. Thus, in the co-evolutionary arms race between predators and prey, toad tadpoles seem to have an advantage over some, but not all predators in breeding ponds (**Paper III**). Further experiments are needed to explicitly test the effectiveness of bufadienolides against other threats (e.g. pathogens or cannibalism).

Our results also suggest that changes in toxin content during development may be consequences of adaptation to predictable variation in predation risk during ontogeny, and therefore represent constitutive age-dependent changes in defence against predators (**Paper I**).

Importantly, our findings are likely to reflect the outcome of concurrent natural selection because we observed inducible changes in toxin synthesis manifesting in the same environment in which study organisms experienced cues on predation threat, and also because the observed changes were induced by predators that co-occur with common toad tadpoles in natural populations (**Paper IV**).

Unanswered remains however the question of how costly ultimately induced chemical defences are and how their expression trades off against other important life-history traits (**Papers I** and **III**). After all, costs are often challenging to demonstrate (DeWitt *et al.*, 1998; Pigliucci, 2005), partly because they do not necessarily appear synchronously with the plastic response, and especially so in species with complex life histories, such as amphibians (Tollrian & Harvell, 1999; Van Buskirk & Saxer, 2001; Benard, 2004; Steiner, 2007).

Collectively, our studies highlight the diversity of factors which can influence the expression and effectiveness of vertebrate chemical defences, such as the co-evolutionary history of predators and their chemically defended prey, the sensitivity of predators to toxins, as well as exposure of prey to environmental stimuli other than predation (see also Bókony *et al.*, 2016; Bókony *et al.*, 2017). Therefore, to detect plastic chemical defences, it is necessary to consider the implications of this complexity. Future studies should focus on explicitly testing the combined effect of conspecifics and predatory cues on toxin synthesis and the role of synergistic selection in shaping induced animal chemical defences, as well as the effectiveness of bufadienolides in defence against pathogens *in vivo* (but see Ujszegi *et al.*, 2017).

Összefoglalás

A fenotípusos plaszticitás a genotípus azon képessége, hogy különböző környezetekben különböző fenotípusokat hozzon létre (West-Eberhard, 1989; Futuyma, 1998; West-Eberhard, 2003; Pigliucci, 2005). A jelenség széles körben elterjedt a természetben és egyre jobban ismert és elismert, hogy milyen alapvető hatása van különböző ökológiai és evolúciós folyamatokra (Harvell, 1990; Tollrian & Harvell, 1999; Agrawal, 2001; West-Eberhard, 2003; Miner *et al.*, 2005; Fordyce, 2006; Pfennig *et al.*, 2010). A fenotípusosan plasztikus válaszok egy speciális esete az indukálható védekezés (Harvell, 1990; Tollrian & Harvell, 1999), amikor a választ az

egyedre nézve potenciálisan veszélyes biotikus környezeti faktorok váltják ki, pl. ragadozók, versenytársak, kórokozók vagy paraziták.

A mérgező anyagok felhalmozása és felhasználása mind ökológiailag, mind evolúciósan fontos adaptáció a fajok közötti interakciók tekintetében. A toxicitás az élővilág minden doménjében elterjedt (Keeler & Tu, 1991; Singh & Tu, 1996; Mebs, 2001; Brodie, 2009; Fry *et al.*, 2009; Yamaguchi *et al.*, 2011; Casewell *et al.*, 2013; Makarova *et al.*, 2013), és habár jól ismert, hogy például a növények képesek ellenségeik megjelenésére méregtermelésük plasztikus megváltoztatásával, vagyis indukált kémiai védekezéssel reagálni (Tollrian & Harvell, 1999; Chen, 2008; McCall & Fordyce, 2010), ezt a jelenséget az állatok esetében még alig vizsgálták (Hettyey *et al.*, 2014).

Mivel a kétéltűek klasszikus modellállatai a fenotípusos plaszticitás kutatásának (Miner *et al.*, 2005) és sok fajuk rendelkezik méreganyagokkal (Fig. 1; Toledo & Jared, 1995; Daly, 2003), különösen alkalmas alanyai lehetnek a kémiai védekezés indukálhatóságának felderítésére. A varangyfélék (Bufonidae család) közismerten méreganyagokkal rendelkező állatok (Toledo & Jared, 1995). Mérgük fő összetevői az ún. bufadienolidok (Fig. 1; Flier *et al.*, 1980; Krenn & Kopp, 1998; Mebs *et al.*, 2007; Gao *et al.*, 2010), amelyek kardiotoxikus hatású szteroid származékok (Flier *et al.*, 1980; Pierre & Xie, 2006; Schoner & Scheiner-Bobis, 2007; Lingrel, 2010). Ezek az anyagok számos ragadozótól hatékonyan védik meg a varangyokat már a nagyon korai fejlődési stádiumoktól kezdve (Kruse & Stone, 1984; Henrikson, 1990; Denton & Beebee, 1991; Peterson & Blaustein, 1991; Lawler & Hero, 1997; Gunzburger & Travis, 2005). Ugyanakkor azt is megfigyelték, hogy a varangy ebihalak jelenléte negatív hatással van más fajok egyedeire (Licht, 1967; Wilbur & Alford, 1985), és lehetséges, hogy ezt a jelenséget szintén a bufadienolidok okozzák (Crossland & Shine, 2012). Elképzelhető tehát, hogy ezek az anyagok multifunkcionális vegyületek, amelyeknek ragadozókat riasztó és allelopatikus hatása (Reigosa *et al.*, 2006) is van egyben.

Ennek megfelelően, kísérleteink során főleg arra kerestük a választ, hogy ragadozók illetve versenytársak jelenléte hogyan befolyásolja a kétéltűek méregtermelését. Vizsgáltuk továbbá a kémiai védekezés indukálhatóságának populációk közötti változatosságát, a méreganyagok szintézisének változását az ontogenezis során és megkíséreltük az indukált méregtermelés energetikai költségeinek feltárását is. Modellállatunknak a barna varangy (*Bufo bufo*) ebihalait választottuk (Fig. 2). A varangy ebihalak ragadozók megjelenésére viszonylag gyengén reagálnak morfológiájukat és viselkedésüket tekintve (Laurila *et al.*, 1998; Lardner, 2000; Van Buskirk, 2002), mégis számos ragadozó ellen jól védettek a korai egyedfejlődés folyamán (Henrikson, 1990; Denton & Beebee, 1991), köszönhetően annak, hogy már ekkor

rendelkeznek méreganyagokkal (Mebs *et al.*, 2007; Bókony *et al.*, 2016; Bókony *et al.*, 2017; Ujszegi *et al.*, 2017). Ez arra enged következtetni, hogy nagymértékben méreganyagaikra támaszkodnak a potenciálisan veszélyes biotikus környezeti faktorok elleni védekezésben. Kísérleteink során a varangy ebihalakat a laborban, illetve kültéri mezokozmoszokban neveltük és manipuláltuk a ragadozókra utaló kémiai ingerek jelenlétét, a versenytársak számát, illetve az elérhető táplálékmennyiséget. A mezokozmoszok önfenntartó kísérleti rendszerek, amelyek közel természetes körülményeket biztosítanak az ebihalaknak (pl. Van Buskirk, 2002; Relyea, 2004; Bókony *et al.*, 2017). Az állatok méreganyagait nagyteljesítményű folyadékkromatográfhoz kapcsolt tömegspektrométerrel (HPLC-DAD-MS) elemeztük. A 2. fejezetben (Chapter 2) található cikkekre és ezek tartalmára félkövér római számokkal hivatkozom (**I** - **IV**).

I. cikk *A kémiai védekezés indukálhatósága és ontogenezise*

A kísérlet során tanulmányoztuk a kémiai védekezés változását az egyedfejlődés során ebihalakban és a metamorfózison átesett fiatal egyedekben, és megvizsgáltuk, hogy a méregtermelés változását az ontogenezis során befolyásolják-e olyan környezeti tényezők, amelyek hatással lehetnek a bufadienolid szintézis költséghatékonyságára. A varangy ebihalakat hármasával tartottuk a laborban és manipuláltuk egyrészt a ragadozókra utaló szaganyagok jelenlétét (avagy a kémiai védekezés szükségességét), másrészt az elérhető táplálék mennyiségét (avagy a kémiai védekezés költségességét), majd az egyedfejlődés során többször méregmintát vettünk. A ragadozókra utaló szagot sebes acsa (*Aeshna cyanea*) lárvák és felnőtt, hím pettyes gőték (*Lissotriton vulgaris*) közösen szolgáltatták.

Azt feltételeztük, hogy ha a varagy ebihalak képesek önálló méregtermelésre, és érzékelik ragadozók jelenlétét, akkor egyedfejlődésük korábbi szakaszában kezdik meg a bufadienolidok termelését és nagyobb mennyiséget szintetizálnak ezekből a vegyületekből a kontroll egyedekhez képest. Mivel a *de novo* méregtermelés vélhetően költséges (Longson & Joss, 2006; Morgenstern & King, 2013), azt prediktáltuk, hogy azok az ebihalak, amelyek csökkentett mennyiségű táplálékot kapnak, kevesebb bufadienolidot fognak termelni, vagy kisebb mennyiségben, mint *ad libitum* etetett társaik.

A varangy embriók csak néhány bufadienolid komponenst tartalmaztak és azokat is csak nagyon kis mennyiségben (**I/**Fig. 2, **I/**Fig. 3). Kelés után az ebihalakban jelenlévő bufadienolid komponensek száma megnőtt és nagyjából változatlan maradt a kísérlet végéig (**I/**Table 2, **I/**Fig. 2, **I/** Table S2), míg a méreganyagok mennyisége a lárvális fejlődés közepéig nőtt, majd a metamorfózis közeledtével csökkent (**I/**Table 2, **I/**Fig. 3, **I/**Table S2). Az

egyedfejlődés első felében azok az ebihalak, amelyek csökkentett táplálékmennyiséghez fértek csak hozzá, szignifikánsan nagyobb összmennyiségű bufadienolidot termeltek *ad libitum* etetett fajtársaikhoz képest (**I/**Table 2, **I/**Fig. 3, **I/**Table S3), annak ellenére, hogy testtömegük jelentősen kisebb volt (**I/** Fig. S2). A ragadozók jelenlétére utaló szag nem indukált változást az ebihalak kémiai védekezésében (**I/**Table 2, **I/**Fig. S4).

Cikkünk explicit módon mutatta ki, hogy a kétéltű ebihalak képesek aktív méregtermelésre, amelyet a kelés utáni megnövekedett komponensszám és össz-méregmennyiség bizonyít. A bufadienolidok összmennyiségének egyedfejlődés során megfigyelt mintázata (növekedés a lárvális fejlődés közepéig, majd azt követően csökkenés) jól illeszkedik általánosságban az ebihalak ragadozókkal szembeni védettségének változásához. Amikor az ebihalak fiatalok, könnyű prédát jelentenek számos ragadozó számára, míg később megnövekedett testméretük és/vagy úszási képességeik révén nagyobb fokú védettséget élveznek mérgek nélkül is (Semlitsch & Gibbons, 1988; Richards & Bull, 1990; Eklöv & Werner, 2000; Wilson & Franklin, 2000). Ugyanakkor lehetséges, hogy a lárvális fejlődés végén látható méregmennyiség csökkenésének az átalakulás megkezdéséhez (Beck & Congdon, 2003; Brown & Cai, 2007; Orlofske & Hopkins, 2009) vagy a ragadozók azon képességéhez is köze van, hogy a kémiailag védett ebihalakkal történő korai találkozás után megtanulják elkerülni őket (Brodie & Formanowicz, 1981; Kruse & Stone, 1984; Nelson *et al.*, 2011), így az ebihalak fejlődésük előrehaladtával mentesülnek a megnövekedett méregtermelés "terhétől". Kísérletünk ezen kívül példát szolgáltat a táplálékmennyiség által indukált megnövekedett méregtermelés jelenségére is. Ez az eredményünk arra enged következtetni, hogy a bufadienolidok termelése energetikailag kevéssé költséges (Kurali *et al*., 2016), habár megjegyzendő, hogy a fitnesz szempontjából az indukált védekezés költséghatékonysága fontosabb lehet, mint a plasztikus válasz abszolút energetikai igénye. Lehetséges, hogy a lecsökkent táplálékmennyiséget az ebihalak a versengés erősödésének jeleként fogták fel és azért kezdtek fokozott méregtermelésbe, hogy versenytársaikat elnyomják vagy védekezzenek azok kórokozói és parazitái ellen. Arra vonatkozó eredményünk, hogy a ragadozók nem indukáltak változást a bufadienolidok termelésében, meglepő, ugyanakkor egyezik egy előző vizsgálatunk eredményével, amely azt mutatta ki, hogy természetes populációkban a méregmennyiség nem korrelál a ragadozók egyedsűrűségével (Bókony *et al.*, 2016).

II. cikk *Versenytársak által indukált kémiai védekezés*

Azért, hogy kísérletesen is teszteljük, hogy a versenytársak jelenléte megnövekedett méregtermelést indukál-e varangy ebihalakban és megvizsgáljuk, hogy az ebihalak mérgének

van-e allelopatikus hatása, azaz arra használják-e ezeket a vegyületeket, hogy elnyomják versenytársaikat (Crossland and Shine, 2012), kültéri mezokozmosz kísérletben manipuláltuk az ebihalak egyedsűrűségét. A kísérletben az ebihalakat fajtársak és erdei béka (*Rana dalmatina*) ebihalak különböző denzitásain neveltük együtt.

Ebben a kísérletben a következő feltételezéseket teszteltük: (1) az erősebb versengés fokozott méregtermelést indukál, (2) az erdei béka ebihalak jelenléte nagyobb mértékben befolyásolja a méregtermelést, mint a fajtársaké, és (3) a varangy ebihalak hátráltatják az erdei béka ebihalak növekedését és fejlődését.

A bufadienolid vegyületek száma marginálisan nem szignifikánsan növekedett a fajtársak egyedsűrűségének növekedésével, ugyanakkor az erdei békák egyedsűrűségével nem állt összefüggésben ez a válaszváltozó (**II/**Table 1, **II/**Fig. 1D). A versenytársaknak ugyanakkor jelentős hatása volt a bufadienolidok összmennyiségére: a méregmennyiség nőtt a fajtársak denzitásának növekedésével, miközben az erdei béka ebihalaknak csak marginálisan nem szignifikáns hatása volt: negatívan befolyásolták a bufadienolid mennyiséget, ha kevesen voltak és növelték, ahogy a számuk nőtt (**II/**Table 1, **II/**Fig. S1). A testtömegre korrigált bufadienolid-összmennyiség is szignifikánsan nőtt a fajtársak számának növekedésével (**II/**Fig. 1F), míg az erdei béka ebihalaknak nem volt ehhez hasonló hatása (**II/**Table 1). A varangyok jelenléte nem gyakorolt negatív hatást az erdei béka ebihalakra, mert sem testtömegük (**II/**Table 1, **II/**Fig. 1C), sem fejlettségük (**II/**Table 1, **II/**Fig. 2) nem függött a velük együtt nevelt varangy ebihalak denzitásától.

Eredményeink azt mutatják, hogy az ebihalak méregmennyisége (és kisebb mértékben a bufadienolidok száma) elsősorban a fajtársak hatására növekedett meg. Ezek alapján, a bufadienolidok az intraspecifikus kommunikáció ágensei lehetnek (Hagman & Shine, 2009; Crossland & Shine, 2011), esetleg a fajtársak közötti aggresszió és kannibalizmus (Blaustein, 1988), vagy pedig a kórokozók és paraziták kivédésében lehet szerepük (Cunha Filho *et al.*, 2005; Tempone *et al.*, 2008; Conlon *et al.*, 2009). Egy másik hipotézis szerint a varangy ebihalak szinergisztikus szelekció következtében (Maynard Smith, 1982; Maynard Smith, 1998; Corning & Szathmáry, 2015), a ragadozók hatékonyabb elriasztása érdekében termelnek nagyobb mennyiségű mérget fajtársak jelenlétében (Sillén-Tullberg & Leimar, 1988; Tullberg *et al.*, 2000). A bufadienolidok allelopatikus hatásának hiánya valószínűleg e vegyületek kémiai tulajdonságaira és a két faj viselkedésbeli különbségeire vezethető vissza. A bufadienolidok amfifil molekulák, ezért alacsony vízoldékonyságúak, így a legnagyobb koncentrációban közvetlenül a bőr és a víz határán lehetnek jelen (Kubanek *et al.*, 2002). Emellett a varangyok aktívabbak és hajlamosabbak a csoportosulásra, mint az erdei béka ebihalak (pers. obs.). A két

faj között létrejövő közvetlen testi kapcsolat és így a méreganyagok átadásának ritkasága eredményezhette azt, hogy az erdei béka ebihalakra nem hatott hátrányosan a barna varangy ebihalak jelenléte.

III. cikk *A kémiai védekezés indukálhatósága különböző ragadozók által*

Ebben a kísérletben azt vizsgáltuk, hogy az ebihalak képesek-e méregtermelésüket négy olyan, filogenetikailag egymástól távol eső ragadozó faj egyedeinek jelenlétéhez igazítani, amelyek veszélyességükben is nagymértékben eltérnek egymástól. Emellett felmértük a ragadozókra adott válasz adaptív voltát is. E célok elérése érdekében az ebihalakat kültéri mezokozmoszokban neveltük, ketrecbe zárt ragadozók jelenlétében, vagy hiányában, meghatároztuk bufadienolid tartalmukat, majd szabadon úszó ragadozóknak tettük ki őket. Ragadozóink a következők voltak: sebes acsa lárva (*A. cyanea*), hátonúszó poloska imágó (*Notonecta* sp.), juvenilis háromtüskés pikó (*Gasterosteus aculeatus*) és felnőtt, hím pettyes gőte (*L. vulgaris*). Adatokat gyűjtöttünk az ebihalak fontos életmenet-jellemzőiről is, úgymint viselkedésükről, testsúlyukról, morfológiájukról és az ebihal stádium hosszáról.

Feltételeztük, hogy a ragadozókkal nevelt ebihalak nagyobb számú és/vagy nagyobb mennyiségű bufadienolidot fognak termelni kontroll társaikhoz képest, hogy a válasz ereje arányos lesz az adott ragadozó veszélyességével, valamint hogy a ragadozók által indukált fenotípussal rendelkező ebihalak jobb eséllyel élik túl a szabadon úszó ragadozókkal való találkozást, mint kontroll társaik.

A ragadozók nem indukáltak változást az ebihalak méregtermelésében (**III/**Table 2, **III/**Fig. 2, **III/**Table S1, **III/**Fig. S1), de azok az ebihalak, akik nehezebbek voltak és a halak jelenlétében nevelkedtek, kevesebb bufadienolid komponenst tartalmaztak, mint ami a kontroll minták száraztömegének és vegyületeik számának allometrikus kapcsolatából következett volna (**III/**Table 2, **III/**Table S2, **III/**Fig. S2). A kísérlet alátámasztotta az **I. cikk**ben kapott eredményeket, hogy ebihal korban az egyedek több mérget termeltek, mint az átalakulás kezdetekor (**III/**Fig. 2). Kezeléseink nem voltak hatással az egyedek túlélésére, viselkedésére, testsúlyára, sem morfológiájára (lásd Supplementary Information for **Paper III**). Azok az ebihalak azonban, amelyek halakkal együtt nevelkedtek, szignifikánsan előbb kezdték meg a metamorfózist, mint a kontroll egyedek (**III/**Fig. S3). A többi ragadozó nem volt hatással a lárvális fejlődés hosszára (lásd Supplementary Information for **Paper III**). Szabadon úszó szitakötő lárva jelenlétében a ketrecbe zárt szitakötő lárvával együtt nevelkedett ebihalak szignifikánsan magasabb arányban éltek túl kontroll fajtársaikhoz képest, a többi ragadozó esetében azonban nem tapasztaltunk ilyen hatást (**III/**Table 3, **III/**Fig. 3, **III/**Table S3).

Kísérletünk fő eredménye megegyezik az **I. cikk**ben találtakkal, mégpedig, hogy a ragadozók nem indukáltak változást az ebihalak méregtermelésében. Két környezeti hatás hátráltathatta, hogy felismerjük a ragadozó indukált méregtermelést a vizsgálataink során. Egyrészt, a genetikai asszimiláció jelenségének révén eredetileg plasztikus jellemzők fixálódhatnak olyan populációkban, ahol egy adott környezeti tényező folyamatosan jelen van (West-Eberhard, 2003; Crispo, 2007; Pfennig *et al.*, 2010). Kísérleteinkhez egy-egy halastóban petéző populációból gyűjtöttük be az állatokat, így lehetséges, hogy a halak folyamatos és sok generációra visszanyúló jelenléte miatt a méregtermelés szintje itt konstitutívvá vált. Másrészt elképzelhető, hogy a fajtársak nagy egyedsűrűsége a ragadozó kezeléstől függetlenül olyan intenzív bufadienolid termelést indukált, hogy a ragadozók elleni további méregmennyiség növekedés vagy feleslegessé, vagy élettanilag lehetetlenné vált. Mivel nem sikerült fenotípusosan plasztikus jelleget kimutatnunk a szitakötő jelenlétében nevelkedett ebihalak esetében, úgy gondoljuk, hogy az ilyen egyedek megnövekedett túlélési esélye a szabadon úszó ragadozóval való találkozáskor valamely, a kísérletünkben nem vizsgált jellemző indukálásával függ össze (pl. megnövekedett aggregációs hajlam, vagy nem bufadienolid típusú méregkomponensek szintézisének fokozása révén).

IV. cikk *Ragadozó indukált kémiai védekezés különböző populációkban*

Azért, hogy felmérjük a ragadozók által indukált kémiai védekezés populációk közötti változatosságát, elvégeztünk egy laborkísérletet, amelyhez 6 különböző élőhelyről, 3 állandó vizű halastóból és 3 időszakos kisvízből gyűjtöttünk be varangy petéket. A kikelő ebihalakat egyesével, ragadozó jelenlétére utaló kémiai ingerek jelenlétében vagy hiányában neveltük, majd 20 nap elteltével mértük bufadienolid-tartalmukat. A ragadozók jelenlétét az ebihalak vizébe juttatott olyan keverékkel szimuláltuk, amelyhez homogenizált fajtársak szagát és ragadozók tartóvizét elegyítettük. Ehhez három ragadozófaj egyedeit használtunk a kísérletben: óriás szitakötő lárvákat (*Anax imperator*), ivarérett csapó sügéreket (*Perca fluviatilis*) és felnőtt, hím pettyes gőtéket (*L. vulgaris*).

Azt feltételeztük, hogy a ragadozókkal szemben naiv ebihalakhoz képest a ragadozószagnak kitett ebihalakban megnövekedett bufadienolid tartalmat találunk, valamint hogy a megfigyelt változás mértéke függeni fog a ragadozó fajától is. Az egyes élőhelyeken uralkodó eltérő predációs veszély miatt lokális adaptációra is számítottunk (Kawecki & Ebert, 2004), egyrészt a bufadienolid szintézis alapszintjének (tehát a ragadozószag-mentes környezetben nevelt ebihalak méregtermelésének mértékének) élőhely típusok közötti különbségeiben, és/vagy a ragadozókkal szemben mutatott méregtermelésbeli válaszreakciók

erősségében. Korábbi vizsgálatok kimutatták, hogy az olyan populációkban, ahol állandóan magas a predációs nyomás, mint pl. állandó vizű élőhelyeken a rablóhalak jelenléte miatt, az indukált morfológiai és viselkedésbeli változások mértéke magasabb lehet az időszakos kisvizekhez képest, ahol kisebb az esélye annak, hogy az egyedek ragadozó áldozatául esnek (Magurran, 1990; Åbjörnsson *et al.*, 2004; Kishida *et al.*, 2007; Herczeg *et al.*, 2010; Hettyey *et al.*, 2016). Másrészt azonban a konstans magas predációs nyomás ahhoz is vezethet, hogy csökken a plaszticitás mértéke az adott populációban (West-Eberhard, 2003; Crispo, 2007; Pfennig *et al.*, 2010). Ennek megfelelően az indukált bufadienolid szintézis tótípusok (állandó vs. időszakos) közötti változatosságára nem tudtunk egyértelmű predikciót felállítani.

A különböző ragadozószag-kezelésnek alávetett ebihalak szignifikánsan több féle bufadienolid vegyületet termeltek szignifikánsan nagyobb összmennyiségben, mint a kontroll kezelésben nevelt fajtársaik (**IV**/Fig. 2, **IV**/Fig. S1). A sügérek szimulált jelenlétében nevelt ebihalak termelték a legtöbb bufadienolidot és a legváltozatosabb méregkoktélt, míg a gőték és szitakötők hatására a mérgek száma és mennyisége köztes értékeket ért el (**IV**/Table S3, **IV**/Fig. S1). A bufadienolid-termelés alapszintjének vizsgálata, a kontroll ebihalak összevetése révén, nem tárt fel szignifikáns különbségeket sem az egyes populációk, sem a két tótípus között (**IV**/Fig. 1). A bufadienolidok számának ragadozók által indukált változatosságát tekintve szignifikáns különbségeket találtunk a populációk között (**IV**/Fig. 2). Amikor a méreganyagok számának változását a három állandó vizű és a három időszakos tóból származó populációk összevonásával elemeztük, szignifikáns változást tapasztaltunk mindhárom ragadozó jelenlétében, mindkét tótípusban külön-külön, és a változás mértéke nem különbözött a két tótípus között (**IV**/Table 1). A bufadienolidok összmennyiségének változásában nem találtunk populációk közötti különbséget (**IV**/Fig. 2). A méreg összmennyiségét tekintve a három állandó vizű és három időszakos tóból származó populációk összevonásával történt elemzés során nem találtunk szignifikáns változást a gőtékkel szembeni válaszban egyik tótípus esetén sem és ugyanez volt a helyzet az állandó vizű tavak esetében a szitakötőkkel szemben. Másrészről azonban halak jelenlétére mindkét tótípusból származó ebihalak szignifikánsan megnövelt össz-méregmennyiséggel válaszoltak (**IV**/Table 1, **IV**/Fig. 2). A méregmennyiség változásának intenzitása egyik ragadozó esetében sem különbözött a kétféle víztest között (**IV**/Table 1).

Kísérletünk során tehát sikerült ragadozó által indukált kémiai védekezést kimutatnunk. Ez az eredményünk ellentmond az **I.** és **III. cikkben** leírt eredménynek. Ezt az ellentétet éppen a méregtermelés indukálhatóságának populációk közötti különbségei magyarázhatják (amennyiben az első két kísérletünkben kevéssé plasztikus populációt vizsgáltunk; Magurran, 1990; West-Eberhard, 2003; Åbjörnsson *et al.*, 2004; Crispo, 2007; Pfennig *et al.*, 2010;

Hettyey *et al.*, 2016), vagy a **IV. cikkben** alkalmazott kezelésenkénti nagyobb mintaszámból eredő nagyobb statisztikai erőre vezethető vissza. Arra a hipotézisre, hogy a plasztikusság mértéke a tótípusok között változna, pl. a predációs nyomásban a két tótípus között tapasztalható eltérések miatt, nem sikerült egyértelmű bizonyítékot találnunk. Hátráltathatja a lokális adaptációt, ha génáramlás van a tavak és más, közeli víztestek között (Kawecki & Ebert, 2004; Yeaman & Otto, 2011; Blanquart *et al.*, 2012). Emellett a tavak térbeli szerkezete, pl. sekély területek megléte alkalmas búvóhelyeket biztosíthat a kétéltűlárvák számára e ragadozókkal szemben, ezáltal nagymértékben csökkentve a halak által kifejtett szelekciós nyomást és az élőhelyek közötti különbségeket.

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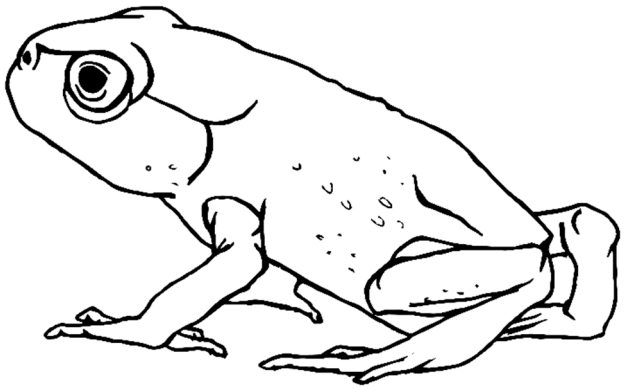
Chapter 2

Original reprints and manuscripts of scientific papers composing the dissertation



Juvenile common toad © *Viktória Verebélyi*

List of papers

I **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; doi: 10.1186/s12862-017-0956-5 (D1, IF = 3,406)

II 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 (D1, IF = 5,210)

III **Üveges, B.**, Szederkényi, M., Mahr, K., Móricz, Á. M., Krüzselyi, D., Bókony, V., Hoi, H., Hettyey, A. (2018): Chemical defence of toad tadpoles under risk by four predator species. Manuscript.

IV Hettyey, A., **Üveges, B.**, Móricz, Á. M., Drahos, L., Capon, R. J., Van Buskirk J., Tóth, Z., Bókony, V. (2018): Predator-induced chemical defence in a vertebrate: toad tadpoles boost their toxin production in response to carnivores. Manuscript.

Own contribution to papers

Phase	I	II	III	IV
Study design	✓	✓	✓	✓
Data collection		✓	✓	✓
Statistical analysis	✓		✓	✓
Manuscript preparation & review process	✓	✓	✓	✓

Original reprints and manuscripts

Paper I

Summary

Chemical defences are widespread in animals, but how their production is adjusted to ecological conditions is poorly known. Optimal defence theory predicts that inducible defences are favoured over constitutive defences when toxin production is costly and the need for it varies across environments. However, if some environmental changes occur predictably (e.g. coupled to transitions during ontogeny), whereas others are unpredictable (e.g. predation, food availability), changes in defences may have constitutive as well as plastic elements. To investigate this phenomenon, we raised common toad (*Bufo bufo*) tadpoles with *ad libitum* or limited food and in the presence or absence of chemical cues on predation risk, and measured their toxin content on 5 occasions during early ontogeny.

The number of compounds showed limited variation with age in tadpoles and was unaffected by food limitation and predator cues. The total amount of bufadienolides first increased and later decreased during development, and it was elevated in young and mid-aged tadpoles with limited food availability compared to their *ad libitum* fed conspecifics, whereas it did not change in response to cues on predation risk. We provide the first evidence for the active synthesis of defensive toxin compounds this early during ontogeny in amphibians. Furthermore, the observation of increased quantities of bufadienolides in food-restricted tadpoles is the first experimental demonstration of resource-dependent induction of elevated *de novo* toxin production, suggesting a role for bufadienolides in allelopathy.

Our study shows that the evolution of phenotypic plasticity in chemical defences may depend on the ecological context (i.e. predation vs. competition). Our results furthermore suggest that the age-dependent changes in the diversity of toxin compounds in developing toads may be fixed (i.e., constitutive), timed for the developmental stages in which they are most reliant on their chemical arsenal, whereas inducible plasticity may prevail in the amount of synthesized compounds.

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BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

Age- and environment-dependent changes in chemical defences of larval and post-metamorphic toads

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Abstract

Background: Chemical defences are widespread in animals, but how their production is adjusted to ecological conditions is poorly known. Optimal defence theory predicts that inducible defences are favoured over constitutive defences when toxin production is costly and the need for it varies across environments. However, if some environmental changes occur predictably (e.g. coupled to transitions during ontogeny), whereas others are unpredictable (e.g. predation, food availability), changes in defences may have constitutive as well as plastic elements. To investigate this phenomenon, we raised common toad (*Bufo bufo*) tadpoles with ad libitum or limited food and in the presence or absence of chemical cues on predation risk, and measured their toxin content on 5 occasions during early ontogeny.

Results: The number of compounds showed limited variation with age in tadpoles and was unaffected by food limitation and predator cues. The total amount of bufadienolides first increased and later decreased during development, and it was elevated in young and mid-aged tadpoles with limited food availability compared to their ad libitum fed conspecifics, whereas it did not change in response to cues on predation risk. We provide the first evidence for the active synthesis of defensive toxin compounds this early during ontogeny in amphibians. Furthermore, the observation of increased quantities of bufadienolides in food-restricted tadpoles is the first experimental demonstration of resource-dependent induction of elevated de novo toxin production, suggesting a role for bufadienolides in allelopathy.

Conclusions: Our study shows that the evolution of phenotypic plasticity in chemical defences may depend on the ecological context (i.e. predation vs. competition). Our results furthermore suggest that the age-dependent changes in the diversity of toxin compounds in developing toads may be fixed (i.e., constitutive), timed for the developmental stages in which they are most reliant on their chemical arsenal, whereas inducible plasticity may prevail in the amount of synthesized compounds.

Keywords: Bufadienolide, Food limitation, Phenotypic plasticity, Predation risk, Tadpole, Toxin production

Background

Chemical defences are widespread across the animal kingdom [1, 2] and can serve for deterring predators, parasites, competitors, and pathogens [1–6]. Some species sequester toxic compounds from food or symbionts [4, 6, 7], or obtain them from ambiguous sources [5, 8, 9], while others are capable of de novo synthesizing toxins [3, 4, 10]. However, in species that synthesise toxic compounds themselves, it has remained largely unknown if chemical defences are inducible, i.e. if their production can vary plastically in response to changing environmental conditions [11] and how inducible chemical defences change during ontogeny [12].

Plastic responses are known to evolve under variable environmental conditions and to come with inherent costs [13, 14]. Therefore, induced chemical defences are especially likely to occur in animals that encounter unpredictably varying environments during their lifetime and synthesise toxins de novo, since such synthesis relies

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on a specialized biochemical pathway and associated physiological and anatomical structures and therefore is considered to be costly [15, 16]. On the other hand, optimal defence theory predicts that changes in chemical defences may become constitutive when environmental differences are predictable [14]; for example, if individuals predictably encounter new environments during their life as a consequence of their development. Consequently, in animals that undergo large, predictable changes in their life-history, and thereby become exposed to drastically different environments that also can unpredictably vary in ecologically important characteristics, chemical defences may both have constitutive as well as inducible components, similarly to other types of defences (e.g. [14, 17, 18]).

Among vertebrates, amphibians undergo the most dramatic changes during their post-embryonic development when they metamorphose and leave the aquatic environment to embark on a terrestrial life [19, 20]. Therefore, amphibians are ideal for studies on ontogenetic changes in toxin production and on the inducibility vs. constitutive nature of chemical defences. Also, chemical defences of vertebrates have been most extensively studied in amphibians. While toxin composition of many amphibian species is well known [3, 7, 21], and experiments documenting age-dependent changes in susceptibility to predators are prevalent in the literature (e.g. [22–26]), in-depth studies on ontogenetic changes in the quantity and composition of toxins utilized in chemical defence and on the underlying secretory apparatus are relatively rare [12, 27–32]. Moreover, there are only a handful of studies on phenotypic plasticity in chemical defences in amphibians [12, 33–35], and only in two of these were larvae sampled for toxin content [12, 35]. Direct evidence for inducible chemical defences in larvae is lacking, and the ability of tadpoles to synthesize toxic compounds has not been confirmed [12]. Also, the studies that so far reported plastic changes in toxin composition in amphibians, and in fact in any vertebrate [12, 33], only documented effects of predators experienced in the larval environment on post-metamorphic animals, while the metamorphic transition from the fully aquatic larval stage to the terrestrial form disrupts selective forces acting during the two life-stages and makes these largely independent of each other. Therefore, evidence for adaptive phenotypic plasticity in chemical defences in species synthesising toxins de novo is lacking.

Here we present a study on the ontogenetic changes and environmental dependence of toxin content in early life-stages of the common toad (*Bufo bufo*). We aimed to (1) examine ontogenetic variation in chemical defences in larval and post-metamorphic common toads and (2) investigate if ontogenetic changes in toxin production may be constitutive or induced by environmental conditions that may affect the pay-off of chemical defence. We experimentally manipulated the presence of chemical cues on predation risk (i.e. the need for toxin production) and food availability (i.e. the costliness of toxin production) and repeatedly assessed the toxin content of individuals during early ontogeny. We predicted that if cues on predation risk are present during tadpole development and tadpoles are able to synthesize toxins themselves, they would start producing such compounds earlier on during their ontogeny and in higher quantities compared to predator-naïve conspecifics. Given that de novo toxin synthesis is considered to be costly [15, 16], we also predicted that food restriction would lead to decreased production of defensive chemicals, manifesting in lowered quantities and decreased numbers of compounds compared to well-fed conspecifics. We chose the common toad as the study species, because it displays relatively weak inducible defences during the larval stage in terms of morphology and behaviour [36–38] and appears to be unpalatable to several predator species [39, 40], suggesting heavy reliance on chemical defence. Also, the chemical composition of *Bufo* skin secretions is relatively well known, their main defensive chemicals being bufadienolides and biogenic amines [41–44], and *B. bufo* are known to contain toxins already in the larval stages [41, 45].

Methods

Experimental procedures

In early spring 2013 we collected 10 common toad pairs from a lake in the Pilis Mountains, Hungary (47° 37′ 24.78″ N, 18° 48′ 27.20″ E) and transported them to the experimental station of the Plant Protection Institute (Centre for Agricultural Research, Hungarian Academy of Sciences) in Budapest. We let the pairs spawn in 200-L containers placed outdoors, filled with 60 L of aged tap water and containing twigs as egg-deposition substrates. After egg-laying, we transferred eggs from each clutch to the laboratory, and placed them into dishpans filled with reconstituted soft water (RSW; [46]) to a depth of 2 cm. Temperature was set to 17 °C at the beginning and was allowed to gradually increase to 22 °C by the end of the experiment. We set the lighting to a 13: 11 h light: dark cycle.

Upon hatching, we haphazardly selected four hatchlings of each family and stored them in 70% HPLC-grade methanol, resulting in 40 samples collected at the start of the experiment. Hatchlings were at developmental stage 19 ([20], Additional file 1: Figure S1). We used this sampling to estimate the baseline of bufadienolide content at the start of larval development. We further assigned randomly selected hatchlings in groups of three to 2-L containers filled with 1.5 L RSW, distributed

randomly among treatments. We employed a three-factorial design with two predator-cue treatments (control vs. chemical cues on predation risk), two food level treatments (ad libitum vs. limited food), and four sampling occasions during the larval and early metamorphic life-stages (for details see below). We replicated each combination of predator treatment × food level treatment × sampling occasion 20 times, resulting in a total of 320 experimental units at the start of the experiment. Each family was represented twice in each treatment combination. Containers were arranged in groups of four in a randomized block design, where each block contained tadpoles from one family.

As predators we used five 4th instar larvae of the southern hawker, *Aeshna cyanea*, and five adult, male smooth newts, *Lissotriton vulgaris*. We kept individuals of both species grouped in 5-L containers filled with 3 L of RSW, and fed them daily with 800 mg *B. bufo* and 800 mg agile frog, *Rana dalmatina*, tadpoles each. We prepared stimulus water by mixing the water taken from the tanks in which we housed and fed the predators, and simulated predation risk by transferring 30 ml stimulus water daily to the rearing containers of tadpoles in the predator-cue treatment group, while adding equal amounts of RSW to the containers of the control group.

We fed tadpoles with a finely ground 4:1 mixture of rabbit chow and fish flakes. Tadpoles assigned to the ad libitum food treatment group received a food amount of ca. 12% of their body mass/individual/day; tadpoles in the limited food treatment group received one-third of that amount. We adjusted food quantity by weighing tadpoles to the nearest mg at the sampling occasions (see below, Additional file 1: Figure S2). We changed the water in the tadpoles' rearing containers every third day. Whenever we observed a dead individual, we removed it, but spontaneous mortality remained relatively low during the experiment (110 tadpoles out of 960, 11.46%).

When tadpoles were approaching metamorphosis, we monitored the rearing containers twice a day. When the first tadpole in a container started to metamorphose (appearance of at least one forelimb, developmental stage 42 according to [20]), we removed the other individuals from that container, decreased the water level to 1 dl and slightly tilted the containers to allow the metamorph to leave the water.

After the sampling of hatchlings at the start of the experiment, we took samples for toxin analysis four more times, preserving 40 individuals at each occasion [47]. The second and third samplings took place after 14 and 21 days, when tadpoles reached the median developmental stages of 28 (range = 28–30) and 34 (31–36), respectively. We took a fourth sample when tadpoles reached a median developmental stage of 38 (37–41). The date of this sampling occasion was not specified a priori, but was rather determined based on how developed tadpoles were (the presence of well-formed hind limbs), to account for potential differences in growth rate between treatment groups. We performed a final sampling when individuals completed metamorphosis (complete disappearance of the tail at stage 46, Additional file 1: Figure S1). Each container was sampled once during the entire experiment, by haphazardly selecting and conserving one individual from it. From the 320 experimental containers we therefore collected 320 samples, half of which we later analysed for toxin content. In each treatment × family combination, one container was a priori designated to chemical analysis while the other container was used as a backup; the latter samples were analysed only if we encountered problems during sample preparation for HPLC of the respective a priori sample (21 instances out of 160 samples, 13.13%, [47]). We released adults, unused eggs and all remaining tadpoles, metamorphs and toadlets at the site of collection.

Analysis of toxin content

We used high-performance liquid chromatography with diode-array detection and mass spectrometry (HPLC-DAD-MS) to identify and quantify bufadienolide compounds. We homogenized specimens using a homogenizer (VWR VDI 12) with a dispersing tool (IKA S12 N-7S). After drying samples in vacuo at 45 °C using a rotary evaporator (Büchi Rotavapor R-134), we measured dry weight of samples using an analytical balance (Ohaus Pioneer PA-114) to the nearest 0.1 mg and subsequently re-dissolved samples in 1 ml HPLC-grade absolute methanol, which was further aided by exposing the samples briefly to ultrasound in a bath sonicator (Tesla UC005AJ1). We filtered the samples using nylon syringe filters (FilterBio, pore size = 0.22 μm). We identified compounds as bufadienolides by inspecting the UV spectrum of peaks [27, 33, 45] and by using commercially acquired bufalin, bufotalin, resibufogenin, gamabufotalin, areno- and telocinobufagin (Biopurify Phytochemicals, Chengdu, China), cinobufagin (Chembest, Shanghai, China), cinobufotalin (Quality Phytochemicals, New Jersey, USA) and digitoxigenin (Santa Cruz Biotechnology, Dallas, TX, USA) as standards (Fig. 1). Identification of compounds present in low quantities was further aided by the analysis of a sample obtained from an adult male common toad by gently massaging the parotoid glands.

A single-quadrupole HPLC-MS system (Shimadzu LC-MS-2020) equipped with a binary gradient solvent pump, a vacuum degasser, a thermostated autosampler, a column oven, a diode array detector and a mass analyser with electrospray ionization (ESI-MS) was used. Chromatographic separations were carried out at 35 °C on a C18 2.6 μm column (Kinetex, 100 mm × 3 mm i.d.)

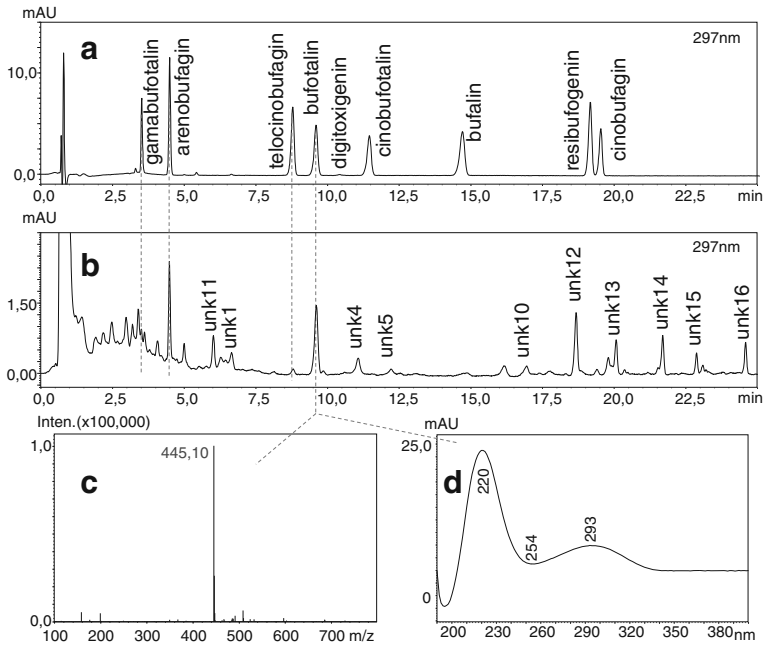

Fig. 1 HPLC-DAD-ESI-MS analysis of bufadienolides. Representative UV chromatograms of the separated standards (**a**) and a common toad sample (**b**, sample nr. 213 [47]), as well as the MS (**c**) and UV (**d**) spectra of bufotalin. Further examples of representative chromatograms of the common toad can be accessed in the online appendix of [45]

in series with a C18 guard column (4 mm × 3 mm i.d.) using 10 μL injections. The mobile phase consisted of water containing 0.05% formic acid (solvent A) and acetonitrile containing 0.05% formic acid (solvent B). The flow rate was 0.8 mL/min and the gradient was as follows: 0–2 min, 15–25% B; 2–15 min, 25–35% B; 15–24 min, 30–50% B; 24–25 min, 50–90% B; 25–30 min 90% B; 30–35 min 15% B. ESI worked under the following conditions: desolvation line (DL) temperature, 250 °C; heat block temperature, 400 °C; drying N_2 gas flow, 15 L/min; nebulizer N_2 gas flow, 1.5 L/min; positive ionization mode. Data was acquired and processed using the programme LabSolutions 5.42v (Shimadzu).

Statistical analyses

To calculate the number of bufadienolide compounds (NBC) present in each animal, we assumed a compound to be present when its area value was larger than zero in the chromatogram (Fig. 1). We estimated the quantity of each compound from the area values of chromatogram peaks (Fig. 1) based on the calibration curve of the bufotalin standard, and summed up these values to obtain an estimate of total bufadienolide quantity (TBQ) for each individual. We used the calibration curve of the bufotalin standard, because this was the most ubiquitously identified compound in our samples (Table 1). This approach yields approximate estimates of bufadienolide quantities, but it has been successfully used before in similar studies [12, 33, 45].

We analysed the effects of predator-cue, food treatments and developmental stage on toxin content using linear mixed-effects models (LMM). We entered NBC or TBQ as the dependent variable; we used the $\log_{10}$-transformed values of TBQ to ensure normality of model residuals and homogeneity of variances. Initial models included food level, predator-cue treatment, and developmental stage of tadpoles as fixed factors, their two-way and three-way interactions; and block nested within family as random factors. In the analyses of TBQ, we also entered the $\log_{10}$-transformed dry mass of tadpoles as a covariate, but without interactions with the other explanatory variables. Note that the data obtained from the first sampling occasion (developmental stage 19) were not included in the LMM analyses, because treatments were only applied after this stage. With each initial model, we performed a backward model-simplification procedure based on *P*-values, with $\alpha = 0.05$. To calculate relevant statistics for non-significant terms that were dropped during model selection, we re-entered the removed variables one by one into the final models. We ran all analyses in R 3.1.3 [48] using the 'lme' function in the 'nlme' package [49]. *P*-values were calculated using 'anova' in 'nlme', using type-3 sums of squares. We conducted pairwise comparisons among treatment groups and samplings by calculating linear contrasts corrected for false discovery rate [50] using the 'lsmeans' package [51]. Model residuals of NBC and TBQ showed

Table 1 Percent occurrence, retention time and mass signal of bufadienolides in common toad tadpoles

Compound name	Percent occurrence of bufadienolide compounds					retention time (min)	m/z $[M + H]^+$
	stage 19	stage 28	stage 34	stage 38	stage 46		
Arenobufagin	10	67.5	90	95	43.6	4.5	417.2
Bufalin	-	25	40	60	56.4	14.5	387.25
Bufotalin	5	97.5	95	97.5	100	9.5	445.3
Gamabufotalin	-	-	15	35	51.3	3.5	403.25
Resibufogenin	-	12.5	5	17.5	15.4	19	385.25
Telocinobufagin	22.5	37.5	47.5	70	100	8.7	403.25
unidentified bufadienolide 1	-	100	100	100	100	6.6	729.35
unidentified bufadienolide 2	2.5	95	92.5	95	100	7.5	727.3
unidentified bufadienolide 3	-	5	-	2.5	51.3	9.6	729
unidentified bufadienolide 4	-	92.5	100	100	100	10.6	715
unidentified bufadienolide 5	-	10	12.5	5	-	11.8	627.4
unidentified bufadienolide 6	-	72.5	72.5	60	94.9	12.3	713.3
unidentified bufadienolide 7	-	12.5	25	2.5	53.8	12.9	671.35
unidentified bufadienolide 8	-	85	90	85	76.9	13	743
unidentified bufadienolide 9	-	75	85	85	-	16.7	671.4
unidentified bufadienolide 10	-	100	85	82.5	97.4	16.9	757.3
unidentified bufadienolide 11	7.5	40	75	87.5	66.7	6	415.3
unidentified bufadienolide 12	2.5	100	97.5	100	56.4	18.6	573.15
unidentified bufadienolide 13	2.5	100	100	100	28.2	20	571.1
unidentified bufadienolide 14	-	100	100	95	82.1	21.7	367.1
unidentified bufadienolide 15	2.5	85	25	10	15.4	22.9	365.1
unidentified bufadienolide 16	-	100	100	97.5	84.6	24.6	601.15

Compounds represented by "-" were not detectable by HPLC-DAD-MS in any of the samples

considerable heteroscedasticity between samplings when developmental stage 19 was included in the analysis (Figs. 2 and 3), therefore in these instances we allowed for different within-sampling variances using 'weights' with 'varIdent' in 'nlme' [49]. We also analysed the quantity of each bufadienolide compound separately; the final models of these analyses are presented in the supplementary information (Additional file 1: Table S1). We had to discard one sample from the analysis on NBC, and two samples from the analysis on TBQ due to missing data [47].

Results

Number of bufadienolide compounds

Toad hatchlings (developmental stage 19) contained a small number of bufadienolides or none at all (median: 0, range: 0-4, N = 40; 25 hatchlings (62.5%) did not contain any compounds in detectable quantities). In contrast, bufadienolides had high diversity in all other age categories (developmental stages 28–46, median NBC: 14, range: 8–20, N = 159; Table 1, Fig. 2). One compound (unidentified bufadienolide 1) was found in all tadpoles and post-metamorphic individuals (Table 1). After hatching, the effect of developmental stage on NBC was marginally non-significant (Table 2), as tadpoles in any stage did not differ from each other significantly, while there was a small but significant difference between post-metamorphic toads and metamorphosing individuals such that the post-metamorphs had slightly fewer (ca. 1 compound less) bufadienolides (Fig. 2, Additional file 1: Table S2). Predation risk and food limitation did not have a significant effect on NBC (Table 2, Additional file 1: Figure S3).

Total bufadienolide quantity

Toad hatchlings contained only minute amounts of bufadienolides (mean ± SE: 12.603 ± 4.065 ng / tadpole, N = 40) compared to all other age categories (mean ± SE: 1555.864 ± 97.796 ng / tadpole, N = 158, Fig. 3). The compound that was present in all individuals after the hatchling stage had the highest mean quantity (unidentified bufadienolide 1; mean ± SE: 313.738 ± 30.424 ng/tadpole). Across the tadpole stages, the total quantity of bufadienolides increased significantly to developmental stage 34 but decreased afterwards (Table 2, Fig. 3, Additional file 1: Table S2). Furthermore, during the

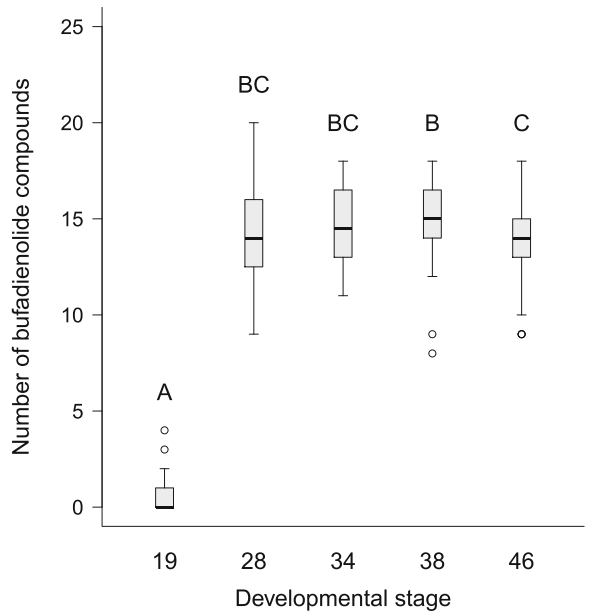

Fig. 2 Number of bufadienolide compounds of common toad hatchlings, tadpoles and post-metamorphs during ontogeny (N = 199). Letters above boxplots indicate homogeneous subsets according to pairwise comparisons corrected for false discovery rate. In each boxplot, the thick horizontal line and the box represent the median and the interquartile range, respectively; whiskers extend to the upper and lower quartile ±1.5 × interquartile range; open circles represent outliers. Statistics for pairwise comparisons can be found in Additional file 1: Table S2

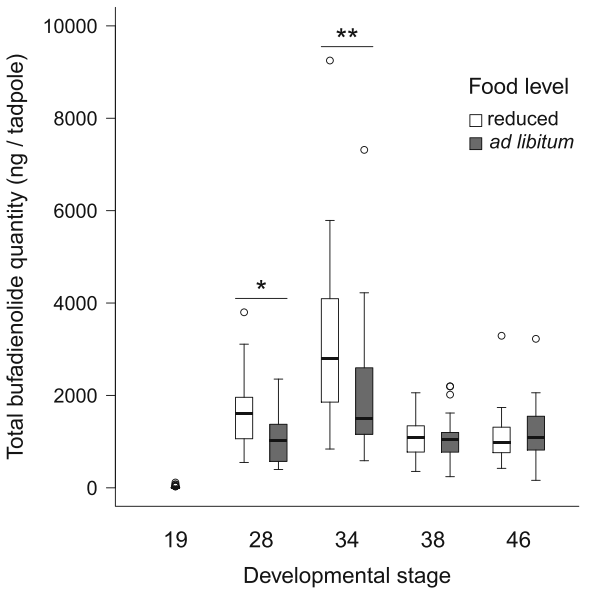

Fig. 3 Total bufadienolide quantity of common toad hatchlings, tadpoles and post-metamorphs (N = 198). Asterisks above boxplots represent pairwise comparisons between food level treatments; groups marked with * (P < 0.05) and ** (P = 0.01) differ significantly based on linear contrasts corrected for false discovery rate. Food level treatment was applied after developmental stage 19 (see Methods). Statistics for pairwise comparisons can be found in Additional file 1: Tables S2 and S3

first half of tadpole development (developmental stages 28 and 34), tadpoles that received reduced amounts of food contained significantly more bufadienolides than their ad libitum fed conspecifics (Table 2, Fig. 3, Additional file 1: Table S3), despite having significantly lower body mass (LMM of body mass, age: $F_{3,\ 214}$ = 160.694, P = <0.0001; food level: $F_{1,\ 214}$ = 26.831, P = <0.0001; age × food level: $F_{3,\ 214}$ = 34.735, P = <0.0001; N = 303; Additional file 1: Figure S2), whereas this difference in TBQ disappeared in more developed tadpoles and post-metamorphic individuals (Fig. 3, Additional file 1: Table S3). Presence or absence of chemical cues on predation risk did not influence TBQ (Table 2, Additional file 1: Figure S4). Analysing the quantity of each bufadienolide compound separately corroborated our findings that toxin content varied with age and food level but not with predation-cue treatment (Additional file 1: Table S1).

Discussion

Our study is the first to unequivocally demonstrate de novo production of toxic compounds in amphibian larvae, as indicated by the steep increase in both the number and quantity of bufadienolide compounds after hatching. This finding shows that common toad tadpoles synthesize their toxins de novo, as has been suggested by histological and ultrastructural studies that demonstrated the presence of the underlying secretory cells and glands already during larval life [30, 31]. This contrasts with other toad species [12, 27] in which tadpoles were found not to produce bufadienolides, relying instead on maternal provisioning of these toxins. For example, in the cane toad (*Rhinella marina*) [27] the diversity and amount of bufadienolides are highest in eggs and gradually decrease until developmental stage 25 [20]. Because we were primarily interested in the phenotypic plasticity of toxin production, we did not investigate eggs, so it remains possible that the same decrease from the egg stage to hatching occurs in common toads. This is supported by observations that common toad eggs are repulsive to many different predator species [39, 40], although compounds other than bufadienolides (e.g. biogenic amines) may also be responsible for the unpalatability of common toad eggs (and hatchlings), as suggested for larvae of *A. boreas* [12]. Nonetheless, because the majority of hatchlings in our study contained no bufadienolides at all, the importance of maternal provisioning of these toxins appears to be limited in common toads. Clearly, maternal toxin provisioning and the temporal changes in toxin content during embryonic development of common toads demands further investigation.

Table 2 Effects of ontogeny, treatments, their interactions, and body mass on bufadienolide synthesis of common toads

	N	df	F	*P*
Number of bufadienolide compounds (NBC)	159			
intercept		**1, 80**	**860.495**	**<0.0001**
developmental stage		3, 80	2.222	0.092
food level		1, 82	0.018	0.894
predation treatment		1, 82	0.442	0.508
developmental stage × food level		3, 76	1.882	0.140
developmental stage × predation treatment		3, 76	0.249	0.862
food level × predation treatment		1, 80	0.266	0.608
developmental stage × food level × predation treatment		3, 68	0.368	0.777
Total bufadienolide quantity (TBQ, ng)	158			
intercept		**1, 78**	**3726.423**	**<0.0001**
body mass		1, 77	2.342	0.130
developmental stage		**3, 78**	**18.313**	**<0.0001**
food level		**1, 78**	**9.646**	**0.003**
predation treatment		1, 77	0.495	0.484
developmental stage × food level		3, 75	2.360	0.078
developmental stage × predation treatment		3, 74	0.358	0.784
food level × predation treatment		1, 76	0.493	0.485
developmental stage × food level × predation treatment		3, 67	0.754	0.524

Terms present in the final models are highlighted in bold. Statistics for non-significant terms that were dropped during model selection were calculated by re-entering the removed variables one by one into the final models

We found that bufadienolides accumulated quickly in young tadpoles and, after reaching a peak in mid-aged larvae, decreased to lower quantities as metamorphosis was approaching. This pattern mirrors ontogenetic changes in tadpole vulnerability: young tadpoles are more vulnerable to predators, thus early toxin production may be strongly favoured. Later, when tadpoles grow larger, they reach a size refuge against several predators or are more difficult to capture [23–26], therefore they may have to rely less on chemical defences. Such an adjustment of toxin dosage to vulnerability to predators may be common in chemically defended organisms [52–54]. Additionally, the ability of predators to distinguish and learn to avoid noxious prey [55–57] may relieve tadpoles from synthesising large amounts of toxins in later stages. Despite a decrease in total bufadienolide quantity, metamorphosing and post-metamorphic individuals in our study still contained considerable amounts of bufadienolides, most likely providing them with effective defences against certain predators and perhaps also pathogens and parasites [58, 59], although metamorphosing anurans (between developmental stages 42 and 46) are more susceptible to predation than late tadpole stages or already metamorphosed animals [60–64]. The decreased bufadienolide quantity we observed in these later stages may be attributed to proximate constraints associated with metamorphosis, when a complete re-organization of several physiological systems occurs [19, 65, 66]. We would expect a similar pattern also in other animal species that actively synthesise toxins and undergo substantial morphological and physiological changes during their ontogeny, such as many insects [67].

The observation that predation risk did not induce the production of larger quantities of bufadienolides in tadpoles is surprising, although it agrees with our earlier finding that, in natural ponds, the toxin content of toad tadpoles did not correlate with the density of predators [45]. A previous experimental study did not observe inducible changes in chemical defences of tadpoles either, but this was attributable to the lack of toxin synthesis in tadpoles of the study species [12]. One possible explanation in our case is that predator-induced changes in chemical defence exist in common toad tadpoles, but not in response to the specific predators we used. However, previous studies did document plastic changes in life-history traits, behaviour and morphology of common toad tadpoles to chemical cues on the presence of *A. cyanea* [38, 68], suggesting that they can detect their presence based on olfactory cues, perceive these predators as dangerous, and respond to them by changes in life-history traits. Because fishes are

considered to be the most voracious predators of amphibian larvae [69], it is possible that in certain populations that live in permanent ponds, such as the one used in the current study, a relatively high baseline level of bufadienolide synthesis becomes fixed via selection [70]. Alternatively, predator-induced plasticity of toxin production may be lacking in toad tadpoles in general, perhaps because of the toxins' apparent low production cost [71], and because very high spatiotemporal variability of predator communities may favour constitutive defences [45]. Finally, although bufadienolides may be effective in repelling several predators, it is possible that the evolution of plasticity in toxin production is driven by other factors, such as pathogens [72, 73] or competitors ([45], see below).

Our results demonstrated inducible changes in toxin production in response to food availability: during early larval life, food-deprived tadpoles contained significantly more bufadienolides than their ad libitum-fed conspecifics. This result, combined with the fact that toxin content was not related to body mass, corroborates our earlier finding that the energetic costs of toxin production in toad larvae may be low [71]. It seems contradictory that an inducible defence may be cheap to produce, but detecting associated costs of expressed plastic traits may be problematic in species with a complex life-history, such as anurans, because costs may not appear synchronously with the displayed trait [74–77]. Nonetheless, enhanced toxin production in food-limited tadpoles concords with results of our field study showing that common toad tadpoles in ponds with high density of competitors (mainly amphibian larvae) contained more bufadienolide compounds and slightly larger total quantities of bufadienolides than tadpoles coexisting with fewer competitors [45]. Thus, in the current experiment, reduced food level might have acted as an indicator of high competitor density, inducing the synthesis of larger amounts of bufadienolides against competitors or the pathogens and parasites they carry. Allelopathy, which is intra- or interspecific competition mediated by chemical substances [78], is a phenomenon of fundamental importance in algae and plants [79, 80], but for animals it has been rarely reported so far [81–83]. The existence of chemical interference between amphibian larvae was proposed long ago, but the mediating agents involved in the process have not been identified [69, 84]. Bufadienolides have been suggested to act as allelochemicals [83], but it remains to be tested directly whether the synthesis of these compounds benefits toad tadpoles by negatively affecting competitors or naturally occurring pathogens and parasites. Nonetheless, our results suggest that allelopathy may be a significant factor in the ecology of a wider variety of animals than currently thought.

Conclusions

In conclusion, our results are the first to document plastic changes in chemical defences in response to food availability in any vertebrate capable of de novo toxin synthesis. The observation that tadpoles produced more toxins at low food availability than when food was present ad libitum indicated that bufadienolides may be relatively cheap to produce but their production may respond plastically to the perceived intensity of competition for food. Our results furthermore suggest that the ontogenetic timing of the production of various toxin components may be fixed as a constitutive defence in toad tadpoles, whereas inducible plasticity prevails in how much of these components is produced. These results, coupled with those of previous studies, highlight the existence of surprisingly diverse strategies of toxin provisioning and synthesis even among as closely related taxa as the species of the Bufonidae family and, thus, caution against premature generalization of observed strategies among species of other chemically defended groups of organisms. Our findings also suggest that ontogenetic changes in toxin production may have resulted from adaptation to predictable variation in predation risk over development, and, thus, represent constitutive age-dependent changes in anti-predator defence rather than a phenotypically plastic response. Therefore, the same trait can show different degrees of phenotypic plasticity depending on evolutionary history (i.e. different species) and ecological context (e.g. predators or other enemies, such as competitors). Studies scrutinizing the costs of toxin production, clarifying the role of toxins in competitive interactions and immune defence, and identifying the environmental factors promoting fixation of the rate of toxin synthesis appear to be especially promising avenues of future research and will provide important insights into the evolution and ecology of chemical defences.

Additional file

Additional file 1: Supplementary methods. Supplementary results. **Tables S1–S4. Figures S1–S4.** (DOCX 238 kb)

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Availability of data and materials
The dataset analysed during the current study is available in the figshare repository, DOI: 10.6084/m9.figshare.4635310, https://figshare.com/s/1e76f47d1f6c3b78a16e [47].

Authors' contributions
BÜ and AH designed the study, GF and AH conducted the experiment and took samples, BÜ prepared samples for chemical analysis, ÁMM and DK analysed samples, BÜ and VB conducted statistical analyses, BÜ, ÁMM, VB and AH wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
All experimental procedures were carried out according to the permits issued by the Közép-Duna-Völgyi KTVF (KTVF: 5192-7/2013) and the Government Agency of Pest County, Hungary (PEI/001/389-4/2013). The experiment was further approved by the Ethical Commission of MTA ATK NÖVI. Consent to participate not applicable.

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Supplementary Information for **Paper I**

Supplementary methods

Statistical analysis of quantity of individual bufadienolide compounds. We considered to analyse variation in toxin diversity using the method proposed by Marion *et al.* (2015), but our experimental design (three fixed effects with multiple levels) rendered such an approach unfeasible. Therefore, to check if our treatments influenced toxin composition, we settled on analysing each bufadienolide's quantity individually, using the analytical procedure described in the main text. Not all compounds could be analysed this way, since in many cases initial models did not meet the assumptions of linearity and/or homoscedasticity. This was especially apparent in the case of rare compounds (13 compounds were absent in more than 25% of tadpoles). Model residuals of some bufadienolides showed considerable heteroscedasticity between treatment groups; in these instances we allowed for different within-group variances using 'weights' with 'varIdent' in 'nlme' (Pinheiro *et al.* 2015). To improve model fit, some compounds' quantities were transformed before analysis as $\log_{10}X$ (if the compound was present in all animals) or $\log_{10}(X+1)$ (if the compound was not detected in some animals). Evaluation of model fit was based on visual observation of diagnostic plots. Statistics of the analysed compounds can be found in Supplementary Table 1.

Supplementary results

Wet body mass of tadpoles. We weighed toad tadpoles to the nearest mg at the sampling occasions right before conserving some of them in methanol. When fed *ad libitum*, tadpoles that received predatory cues had smaller body mass compared to their predator-naïve conspecifics (LMM of body mass, food level × predator cue: $F_{1,214} = 7.764$, $P = 0.006$, $N = 303$, Supplementary Table 4, Supplementary Fig.2). This effect could not be observed in tadpoles that received a reduced amount of food (Supplementary Table 4, Supplementary Fig.2), probably because the amount of food was so limited that tadpoles that received predator cues could not afford to reduce their foraging time without risking starvation, and/or because the full amount of food provided could be ingested even with a reduced activity level.

Citations

Marion, Z.H., Fordyce, J.A. & Fitzpatrick, B.M. 2015. Extending the concept of diversity partitioning to characterize phenotypic complexity. *Am. Nat.* **186:** 348-361.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team 2017. nlme: Linear and nonlinear mixed effects models, The R Project for Statistical Computing. R package version 3.1-131.

Supplementary Table 1: Effects of developmental stage, food level, their interactions, and body mass on the quantity of individual bufadienolide compounds in common toad tadpoles. For brevity, only statistics of the final models are presented. Significant (P<0.05) terms are marked with an asterisk ("†" marks a marginally non-significant interaction obtained by adding it to the final model). Statistics of non-significant terms are available from the authors upon request.

Bufadienolide	*N*	df	F	*P*
	158			
Bufotalin				
intercept *		1, 75	93.891	<0.0001
developmental stage *		3, 75	19.955	<0.0001
food level *		1, 75	10.052	0.0022
developmental stage × food level *		3, 75	8.197	0.0001
Unidentified bufadienolide 1				
intercept *		1, 78	604.166	<0.0001
developmental stage *		3, 78	24.537	<0.0001
food level *		1, 78	22.425	<0.0001
Unidentified bufadienolide 2				
intercept *		1, 75	171.314	<0.0001
developmental stage *		3, 75	8.421	0.0001
food level *		1, 75	10.352	0.0019
developmental stage × food level *		3, 75	4.585	0.0053
Unidentified bufadienolide 4				
intercept *		1, 75	323.246	<0.0001
developmental stage *		3, 75	8.105	0.0001
food level *		1, 75	12.397	0.0007
developmental stage × food level *		3, 75	3.771	0.0141

Supplementary Table 1 continued.

Bufadienolide	*N*	df	F	*P*
Unidentified bufadienolide 6				
intercept *		1, 75	49.137	<0.0001
developmental stage *		3, 75	11.778	<0.0001
food level		1, 75	2.689	0.1053
developmental stage × food level *		3, 75	13.995	<0.0001
Unidentified bufadienolide 8				
intercept *		1, 78	83.388	<0.0001
developmental stage *		3, 78	6.281	0.0007
food level *		1, 78	13.359	0.0005
Unidentified bufadienolide 10				
intercept *		1, 75	293.146	<0.0001
developmental stage *		3, 75	13.944	<0.0001
food level		1, 75	2.230	0.1395
developmental stage × food level *		3, 75	12.236	<0.0001
Unidentified bufadienolide 14				
intercept *		1, 78	88.178	<0.0001
body mass *		1, 78	31.279	<0.0001
developmental stage *		3, 78	94.141	<0.0001
food level		1, 74	0.241	0.625
developmental stage × food level †		3, 74	2.339	0.0804
Unidentified bufadienolide 16				
intercept *		1, 78	104.562	<0.0001
body mass *		1, 78	15.298	0.0002
developmental stage *		3, 78	186.924	<0.0001

Supplementary Table 2: Pairwise comparisons of the number and quantity (ng / tadpole) of toxin compounds between different developmental stages of common toads. Total bufadienolide quantity was $\log_{10}(X+1)$ transformed before analysis. Significant differences (FDR-corrected $P < 0.05$) are marked with an asterisk.

Comparison	Difference	SE	df	*t*	*P*
Number of bufadienolide compounds					
Developmental stage 19 & 28*	-13.575	0.393	185	-34.557	<0.0001
Developmental stage 19 & 34*	-13.975	0.353	185	-39.625	<0.0001
Developmental stage 19 & 38*	-14.275	0.347	185	-41.193	<0.0001
Developmental stage 19 & 46*	-13.17	0.368	185	-35.808	<0.0001
Developmental stage 28 & 34	-0.4	0.492	185	-0.812	0.469
Developmental stage 28 & 38	-0.7	0.488	185	-1.435	0.219
Developmental stage 28 & 46	0.405	0.503	185	0.805	0.469
Developmental stage 34 & 38	-0.3	0.456	185	-0.658	0.512
Developmental stage 34 & 46	0.805	0.473	185	1.704	0.15
Developmental stage 38 & 46*	1.105	0.468	185	2.361	0.039
Total bufadienolide quantity					
Developmental stage 19 & 28*	-2.582	0.114	184	-22.595	<0.0001
Developmental stage 19 & 34*	-2.839	0.116	184	-24.398	<0.0001
Developmental stage 19 & 38*	-2.501	0.112	184	-22.342	<0.0001
Developmental stage 19 & 46*	-2.495	0.117	184	-21.284	<0.0001
Developmental stage 28 & 34*	-0.258	0.056	184	-4.569	<0.0001
Developmental stage 28 & 38	0.081	0.047	184	1.738	0.105
Developmental stage 28 & 46	0.087	0.058	184	1.488	0.154
Developmental stage 34 & 38*	0.339	0.052	184	6.572	<0.0001
Developmental stage 34 & 46*	0.344	0.062	184	5.533	<0.0001
Developmental stage 38 & 46	0.006	0.053	184	0.105	0.916

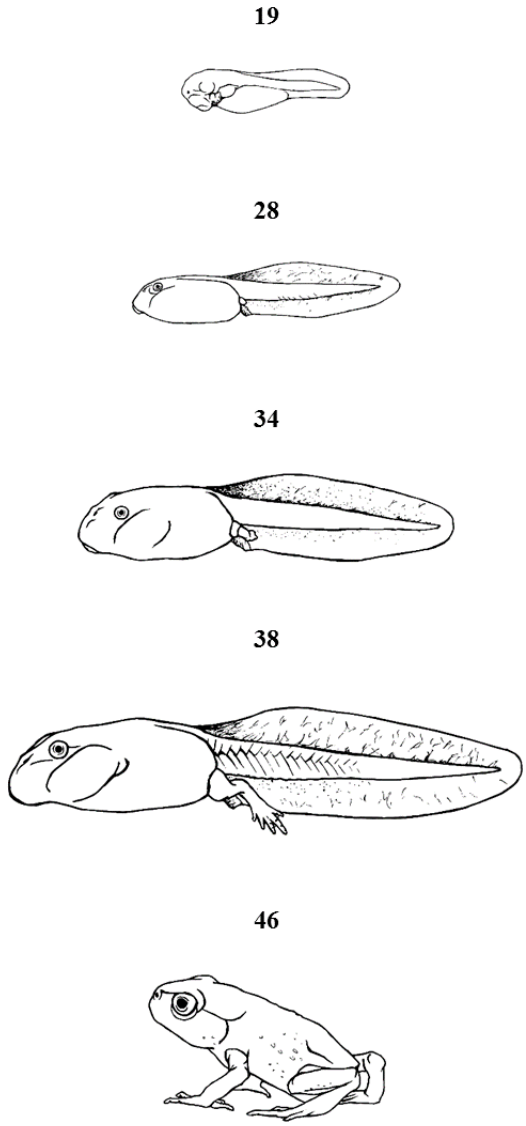
Supplementary Table 3: Pairwise comparisons of total bufadienolide quantity (ng / tadpole) between food levels within developmental stages of common toads. Total bufadienolide quantity was $\log_{10}$-transformed before analysis. Significant terms (FDR-corrected $P < 0.05$) are marked with an asterisk.

Comparison	Difference	SE	df	*t*	*P*
Developmental stage 28, reduced & *ad libitum* food*	0.197	0.074	75	2.657	0.019
Developmental stage 34, reduced & *ad libitum* food*	0.232	0.074	75	3.135	0.01
Developmental stage 38, reduced & *ad libitum* food	0.017	0.075	75	0.224	0.824
Developmental stage 46, reduced & *ad libitum* food	0.019	0.075	75	0.247	0.824

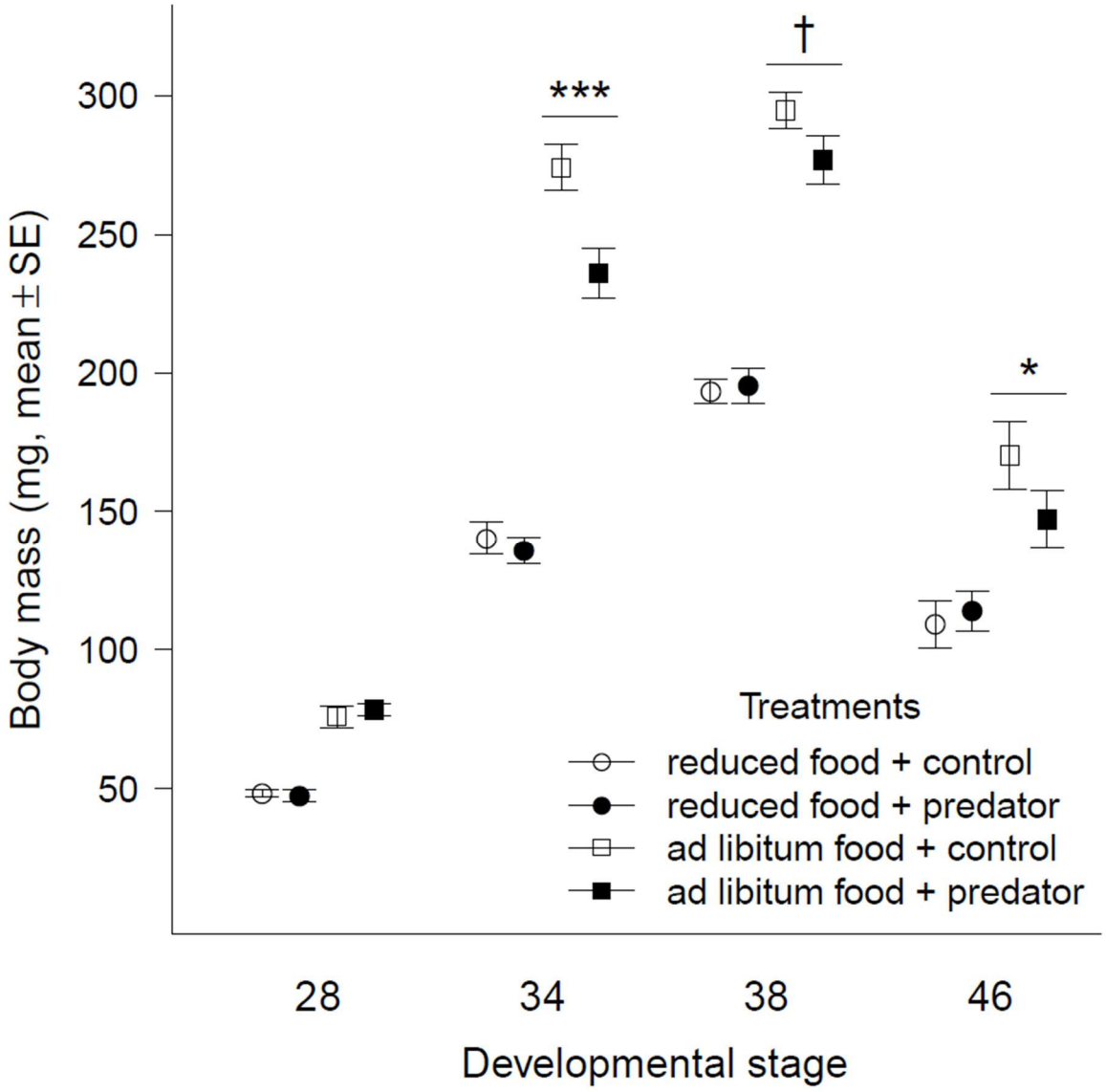
Supplementary Table 4: Pairwise comparisons of wet body mass (mg) of common toad tadpoles between predator cue treatments within developmental stages and food levels. Significant terms (FDR-corrected P < 0.05) are marked with an asterisk ("†" depicts a marginally non-significant difference).

Comparison	Difference	SE	df	*t*	*P*
Developmental stage 28, reduced food, control & predator cues	0.75	9.388	208	0.08	0.936
Developmental stage 34, reduced food, control & predator cues	4.635	9.646	208	0.481	0.631
Developmental stage 38, reduced food, control & predator cues	-1.943	9.646	208	-0.201	0.841
Developmental stage 46, reduced food, control & predator cues	-4.791	9.766	208	-0.491	0.624
Developmental stage 28, *ad libitum* food, control & predator cues	-2.5	9.388	208	-0.266	0.79
Developmental stage 34, *ad libitum* food, control & predator cues *	38.284	9.511	208	4.025	0.0001
Developmental stage 38, *ad libitum* food, control & predator cues †	17.968	9.765	208	1.84	0.067
Developmental stage 46, *ad libitum* food, control & predator cues *	22.883	10.255	208	2.231	0.027

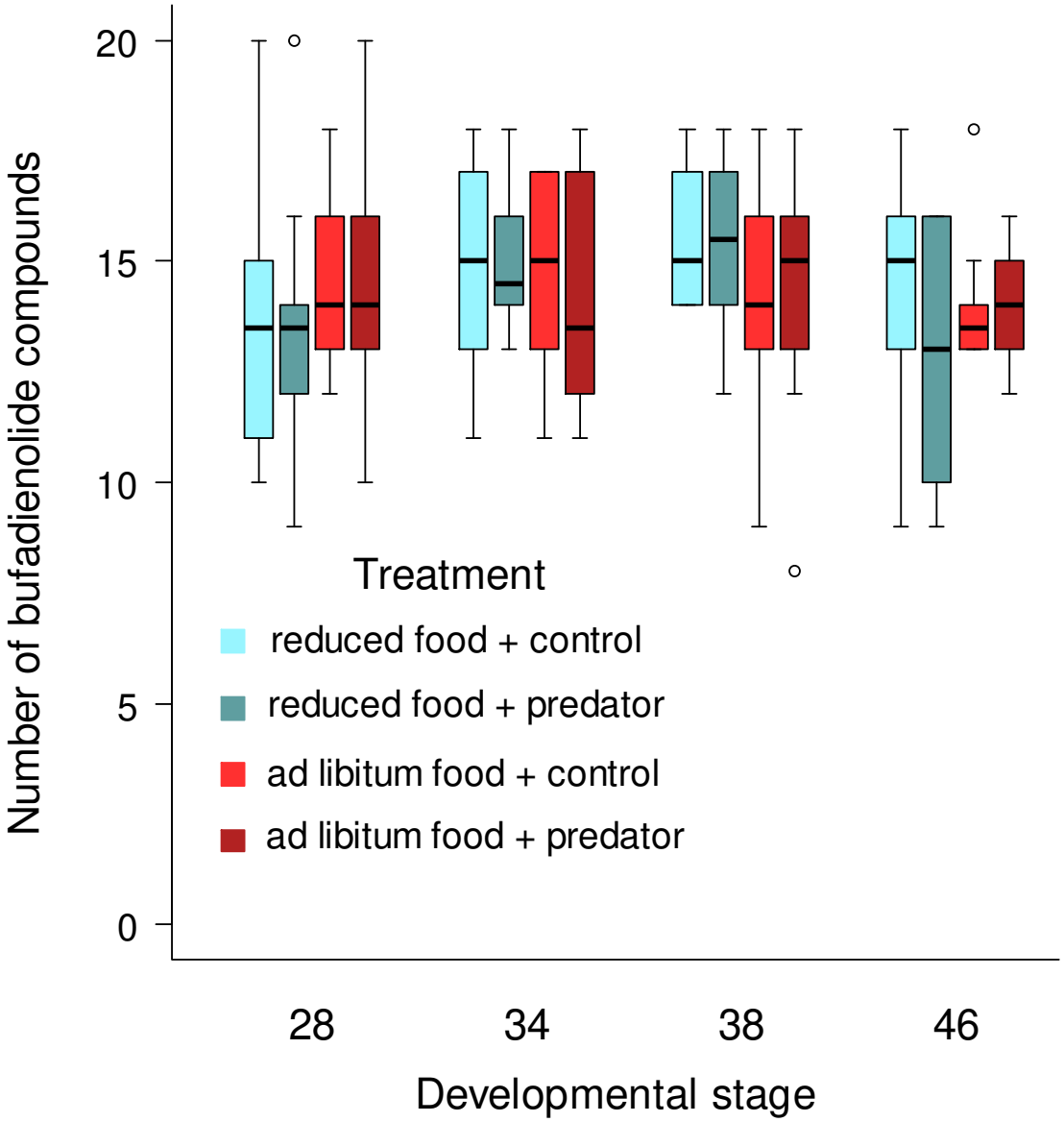
Supplementary Fig. 1: Schematic representation of median developmental stages of common toad tadpoles at the sampling occasions (drawn by Viktória Verebélyi).



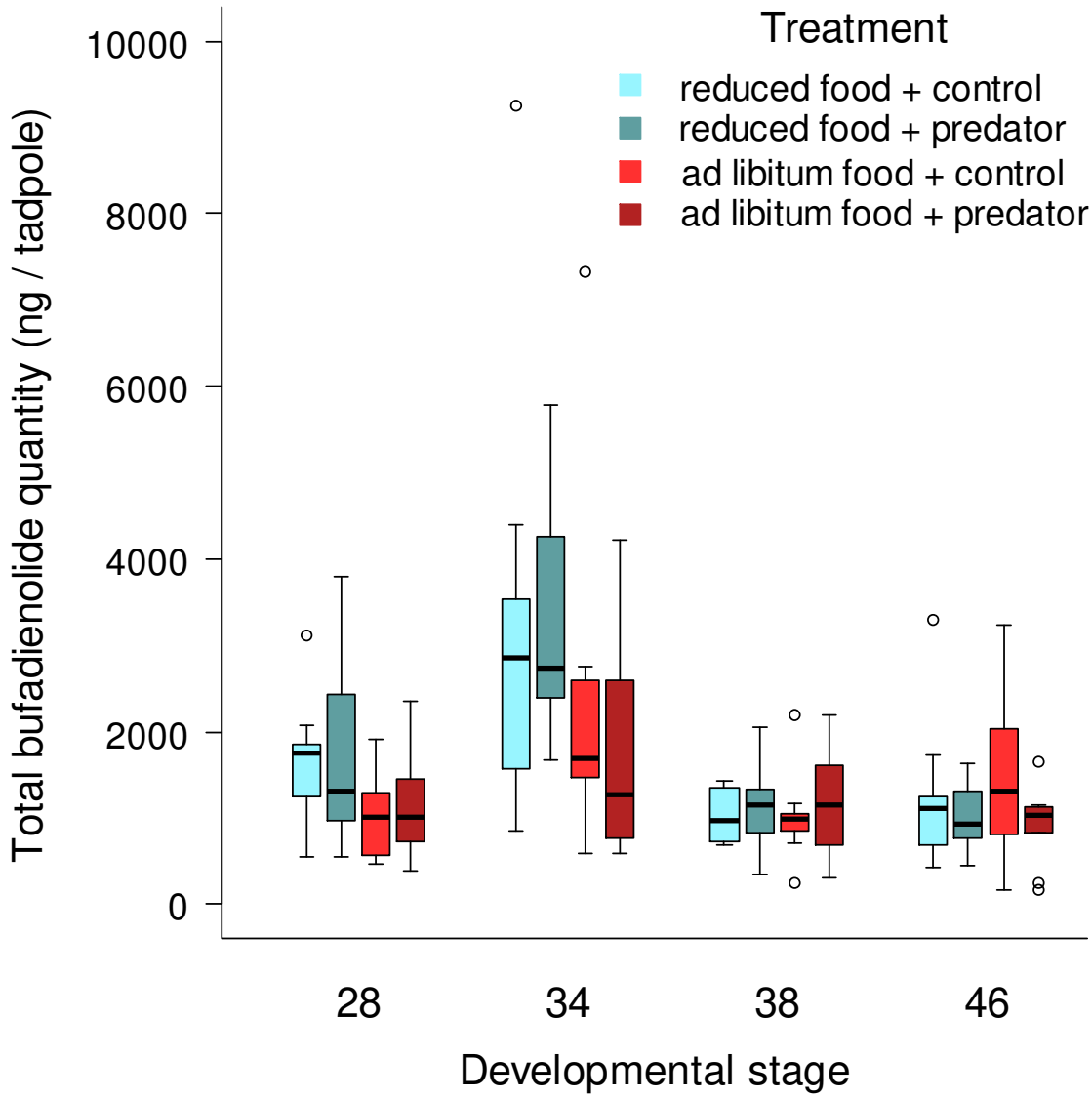
Supplementary Fig. 2: Wet body mass of common toad tadpoles during ontogeny in various experimental treatment groups. Mean ± SE are presented ($N = 303$). Asterisks above error bars represent results of pairwise comparisons; groups marked with * ($P < 0.05$) and *** ($P < 0.001$) differed significantly based on linear contrasts corrected for false discovery rate ("†" marks a marginally non-significant difference, $P = 0.067$).



Supplementary Fig. 3: Number of bufadienolide compounds of common toad tadpoles by developmental stage and experimental treatments (N = 159). 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; open circles represent outliers. Note the lack of predator effects.



Supplementary Fig. 4: Total bufadienolide quantity of common toad tadpoles by developmental stage and experimental treatments (N = 158). Note the lack of predator effects.



Paper II

Summary

Inducible defences are a form of phenotypic plasticity by which organisms respond to and mitigate the threat posed by predators, parasites and competitors. While anti-predatory defences are often in trade-off with anti-competitor responses, chemicals that deter predators may have negative effects on competitors as well. Allelopathy is well known in plants and plant-like animals, but whether the toxins of mobile, behaviourally and morphologically complex animals are induced by and exert allelopathic effects on competitors is poorly known. Common toads *Bufo bufo* synthesize bufadienolides which make them unpalatable or toxic to many predators. However, bufadienolide content of toad tadpoles correlates positively with the density of competitors in natural populations, suggesting that they may upregulate their toxin production to inhibit their competitors, such as heterospecific tadpoles that may be vulnerable to toad toxins. We conducted a microcosm experiment with tadpoles of common toads and agile frogs *Rana dalmatina*, in which we manipulated the density of conspecific and heterospecific competitors. We measured the bufadienolide content of toad tadpoles to test for competitor-induced changes in toxin production, and we assessed the growth and development of agile frog tadpoles to test for allelopathy.

We found that toad tadpoles contained higher amounts of bufadienolides at higher densities; however, heterospecific competitors did not have a stronger effect than conspecifics. Furthermore, the presence or density of toad tadpoles had no effect on the body mass and development rate of agile frog tadpoles.

Our results demonstrate competitor-induced plasticity in toxin production, but we found no support for an allelopathic function of bufadienolides. Instead, we suggest that inducible changes in bufadienolide production may serve to mitigate risks posed by competitors, including aggression, cannibalism or disease. Therefore, bufadienolides are intriguing candidates for multi-purpose defences that may provide protection not only against predators but also against competitors.

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RESEARCH ARTICLE

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Competition induces increased toxin production in toad larvae without allelopathic effects on heterospecific tadpoles

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Abstract

1. Inducible defences are a form of phenotypic plasticity by which organisms respond to and mitigate the threat posed by predators, parasites and competitors. While anti-predatory defences are often in trade-off with anti-competitor responses, chemicals that deter predators may have negative effects on competitors as well. Allelopathy is well known in plants and plant-like animals, but whether the toxins of mobile, behaviourally and morphologically complex animals are induced by and exert allelopathic effects on competitors is poorly known.
2. Common toads *Bufo bufo* synthesize bufadienolides which make them unpalatable or toxic to many predators. However, bufadienolide content of toad tadpoles correlates positively with the density of competitors in natural populations, suggesting that they may upregulate their toxin production to inhibit their competitors, such as heterospecific tadpoles that may be vulnerable to toad toxins.
3. We conducted a microcosm experiment with tadpoles of common toads and agile frogs *Rana dalmatina*, in which we manipulated the density of conspecific and heterospecific competitors. We measured the bufadienolide content of toad tadpoles to test for competitor-induced changes in toxin production, and we assessed the growth and development of agile frog tadpoles to test for allelopathy.
4. We found that toad tadpoles contained higher amounts of bufadienolides at higher densities; however, heterospecific competitors did not have a stronger effect than conspecifics. Furthermore, the presence or density of toad tadpoles had no effect on the body mass and development rate of agile frog tadpoles.
5. Our results demonstrate competitor-induced plasticity in toxin production, but we found no support for an allelopathic function of bufadienolides. Instead, we suggest that inducible changes in bufadienolide production may serve to mitigate risks posed by competitors, including aggression, cannibalism or disease. Therefore, bufadienolides are intriguing candidates for multi-purpose defences that may provide protection not only against predators but also against competitors.

KEYWORDS
allelopathy, amphibian toxins, chemical defence, chemical interference, growth inhibition, growth-defence trade-off, inducible defences, phenotypic plasticity

1 | INTRODUCTION

In response to the risk posed by natural enemies, many organisms including animals and plants produce altered phenotypes that provide protection against those enemies; this form of phenotypic plasticity is referred to as inducible defence (Adler & Harvell, 1990; Tollrian & Harvell, 1999). It occurs in diverse taxa in many forms, including changes in body shape that reduce palatability or enhance escape ability, behavioural responses that reduce the encounter rate with or detectability to predators, and accumulation of repellent or toxic chemicals (Adler & Harvell, 1990; Hettyey, Tóth, & Van Buskirk, 2014; Tollrian & Harvell, 1999). So far, the majority of research on inducible defences has focused on the effects of predators (not counting the extensive research on immune responses to pathogens), demonstrating that predator-induced phenotypic changes are ubiquitous and effective means of enhancing the survival of prey (Adler & Harvell, 1990; Hettyey, Vincze, Zsarnóczai, Hoi, & Laurila, 2011; Relyea & Auld, 2005; Tollrian & Harvell, 1999; Van Buskirk, 2002). However, predators are not the only kind of enemies that organisms need to fend off; competitors can also have large effects (Connell, 1983; Gurevitch, Morrow, Wallace, & Walsh, 1992). The adaptive responses against competitors are often in trade-off with the adaptive responses against predators: for example, behavioural and morphological changes that are beneficial in competition, such as elevated foraging activity and larger intestines which facilitate growth, expose individuals to higher predation risk (Relyea, 2002; Relyea & Auld, 2004, 2005; Tollrian & Harvell, 1999). Chemical defences are particularly intriguing in this respect because they may be multi-functional in the sense that a single phenotype may provide protection against several types of enemies (Hettyey et al., 2014). For example, in plants and soft corals, the defensive chemicals can have both anti-predatory and anti-competitor effects (Kubanek et al., 2002; Siemens, Garner, Mitchell-Olds, & Callaway, 2002). Understanding such responses whose effectiveness against predators and competitors is not traded off against each other (Ramamonjisoa & Natuhara, 2017; Siemens et al., 2002) should provide valuable insights into the ecology and evolution of phenotypic plasticity (Hettyey et al., 2014).

In competitive interactions, organisms can use chemical substances that provide advantage by harming their competitors; such substances have been variably termed defensive or offensive chemicals, allelochemicals or allomones (Berenbaum, 1995). Chemical interference or allelopathy can be an effective way of overcoming competitors, especially in sessile organisms like plants, fungi and benthic marine invertebrates (Reigosa, Pedrol, & González, 2006). The role of allelochemicals in competitive interactions is much less known in mobile animals that can employ a wide diversity of behavioural responses against their foes, although toxins can be found in many of such organisms (Brodie, 2009; Casewell, Wüster, Vonk, Harrison, & Fry, 2013). Defensive toxins of such animals are thought to function mainly as anti-predatory adaptations, and there is some evidence that they can be induced in prey animals by predation threat (Benard & Fordyce, 2003; Hagman, Hayes, Capon, & Shine, 2009) similar to the herbivore-induced chemical responses of primary producers (Tollrian & Harvell, 1999). However, we know very little about the phenotypic plasticity of toxin production in animals in response to competitors (Adler & Harvell, 1990; Hettyey et al., 2014).

In this study, we investigated the effect of competition on the toxin production of amphibian larvae, and the allelopathic potential of competitor-induced toxin production. At high densities, amphibian larvae compete for food by both exploitation and interference (Wells, 2007), and chemical interference has long been suspected as a mechanism by which tadpoles can inhibit the growth of their competitors (Crossland & Shine, 2012; Licht, 1967; Wells, 2007). Despite considerable research effort, however, it is still unclear whether this interference is mediated by specific growth-inhibitor substances, metabolic waste products, or facultative gut parasites such as yeasts or algae (Bardsley & Beebee, 2001; Griffiths, Denton, & Wong, 1993; Wells, 2007). Furthermore, it is not clear how tadpoles could inhibit the growth of conspecifics by such substances without suffering from autotoxicity themselves (Wells, 2007), suggesting that chemical interference is more likely to function in interspecific competition, similar to allelopathy among plants (Reigosa et al., 2006) and to the chemical repellents used by ants for deterring heterospecific competitors from food sources (Adams & Traniello, 1981).

We examined common toads *Bufo bufo*, which contain toxins that make them distasteful or even lethal upon ingestion or contact (Crossland, Brown, & Shine, 2011; Henrikson, 1990) or via indirect, waterborne interactions (Crossland & Shine, 2012; Crossland et al., 2011). Their main toxins are steroid compounds called bufadienolides, which they start to synthesize early during larval development (Üveges et al., 2017). Our earlier studies showed that in common toad larvae, the diversity and quantity of bufadienolides were higher in natural populations with higher competitor density (Bókony et al., 2016) and increased when tadpoles were food-restricted in the laboratory (Üveges et al., 2017); both findings suggested that competition induced toxin production. Toad tadpoles often develop in the same water bodies and live on similar diets as tadpoles of other, non-toxic species, such as agile frogs *Rana dalmatina* (Bókony et al., 2016; McDiarmid & Altig, 1999). Because agile frogs usually start to spawn several weeks before toads in Hungary (Hettyey, Török, & Kovács, 2003) and the tadpoles of the former species grow to larger sizes (Lardner, 2000), toad tadpoles would benefit from inhibiting the growth and development of agile frog tadpoles. Whether such inhibition occurs and whether it is associated with toad toxin levels has not been investigated yet, although other bufonid species were observed to have strong negative effects on other ranid species during larval competition (Alford & Wilbur, 1985; Licht, 1967). Using the common toad–agile frog system, we investigated competitor-induced toxicity and allelopathy by testing the following predictions: (1) stronger competition induces increased toxin production, (2) heterospecific competitors have a larger effect on toxin production than do conspecific competitors and (3) toxin-producing tadpoles inhibit the growth and development of non-toxic heterospecific tadpoles. We experimentally manipulated the strength of competition and the ratio of conspecific and heterospecific competitors in microcosm communities, mimicking natural conditions of small

ponds that are the typical larval habitats of these amphibians (Vági, Kovács, Băncilă, Hartel, & Anthony, 2013).

2 | MATERIALS AND METHODS

2.1 | Experimental design

We raised common toad (henceforth Bufo) and agile frog (henceforth Rana) tadpoles in eight density treatments (Figure 1a) following a response surface design (Inouye, 2001). The densities were chosen to reflect low, medium and high levels of competition based on our previous experience with mesocosm experiments with the two study species (Bókony, Mikó, Móricz, Krüzselyi, & Hettyey, 2017; Hettyey et al., 2011; Mikó, Ujszegi, Gál, Imrei, & Hettyey, 2015). Three treatment groups (6B, 12B and 24B) contained only Bufo tadpoles (Figure 1a) to test if the production of bufadienolides is adjusted to the density of conspecific competitors. Three treatment groups contained tadpoles of both species (Figure 1a) to compare the effects of conspecific competitors to the effects of heterospecific competitors on the production of bufadienolides, while keeping the total biomass constant. The relative numbers of the two species in these treatments were designed based on our observation that Rana tadpoles grow up to twice as large as Bufo tadpoles in outdoor mesocosms. Thus, we expected six Bufo larvae plus three Rana larvae (treatment 6B3R) to have similar total biomass as 12 Bufo larvae (treatment 12B). Similarly, we expected six Bufo larvae combined with nine Rana larvae (treatment 6B9R) to have a total biomass similar to that of 12 Bufo larvae combined with six Rana larvae (treatment 12B6R) or 24 Bufo larvae (treatment 24B). The expected ratio of the two species' biomass was 1:1 in treatments 6B3R and 12B6R, while in treatment 6B9R, it was 1:3 (Bufo:Rana). This latter treatment was added for double purpose: to address not only competition-induced toxicity but also allelopathy, because we

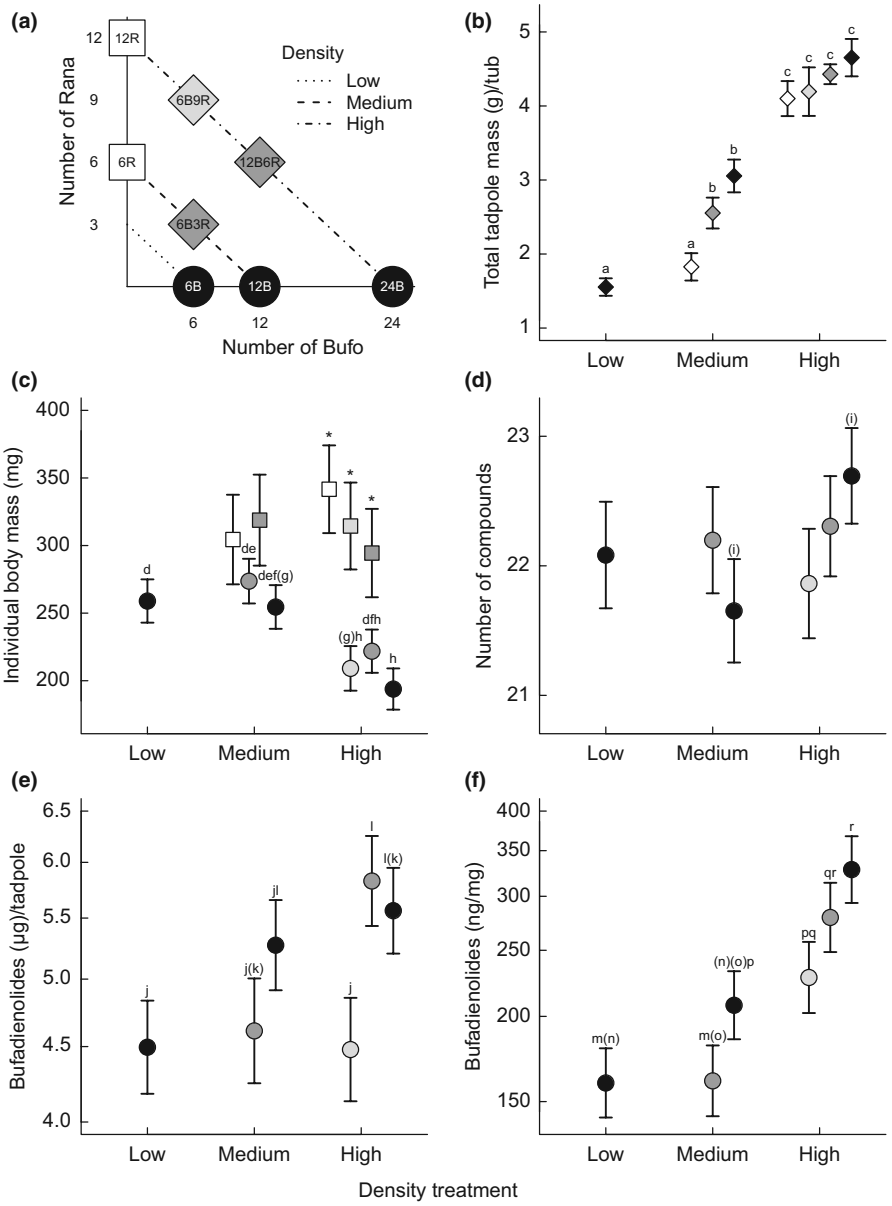

FIGURE 1 Schematics of the experimental design (a), and the effects of density treatments on the *M* ± *SE* of tadpole body mass (b, c) and bufadienolides (d–f). Letters above the error bars indicate homogenous subsets after correction for multiple comparisons, i.e. groups marked by different letters differ significantly from each other ($p < .05$), and letters in brackets indicate marginally non-significant differences (g: $p = .068$, i: $p = .085$, k: $p = .079$, n: $p = .070$, o: $p = .078$). Asterisks above error bars denote significant differences of Rana from Bufo at the same total number of tadpoles. Note the logarithmic scale on the *Y* axis in e and f. Symbol colour denotes the tubs' species composition (black: Bufo only, white: Rana only; dark grey: both species, more Bufo than Rana; light grey: both species, fewer Bufo than Rana); symbol shape denotes the species in which the dependent variable was measured (circles: Bufo, squares: Rana, diamonds: all tadpoles)

expected that 6 Bufo in treatment 6B9R could produce half as much toxin as 12 Bufo in treatment 12B6R, so the Rana tadpoles in these two treatments would experience the same total biomass (high density) but different exposure to toxins. Finally, two treatment groups (6R, 12R) contained Rana tadpoles only (Figure 1a), serving as controls for testing whether Bufo tadpoles inhibit the growth and development of Rana tadpoles. There was only one Bufo tadpole missing at the termination of the experiment possibly due to mortality (in treatment 6B3R). Due to an error, nine instead of six Bufo tadpoles were placed in one tub in treatment 6B9R; however, this tub was not an extreme data point in any of the examined variables (in the analyses we treated this tub as if there had been six Bufo in it, to avoid having a treatment group with $n = 1$). All treatments were started with the same amount of food (see below); we expected the *per capita* food availability to decrease more in treatments with higher density due to exploitation competition, reducing growth.

2.2 | Experimental procedures

In early spring 2016, we collected 60 eggs from each of nine freshly laid Bufo clutches and 30 eggs from each of nine freshly laid Rana clutches from a natural pond in Hungary (47°44′4.12″N, 18°49′7.04″E). We transported the eggs to the experimental station of the Plant Protection Institute in Budapest, where we kept Bufo eggs in 0.5 L and Rana eggs in 1 L reconstituted soft water (RSW; 48 mg $NaHCO_3$, 30 mg $CaSO_4 \times 2\ H_2O$, 61 mg $MgSO_4 \times 7\ H_2O$, 2 mg KCl added to 1 L reverse osmosis-filtered water). Room temperature was 21°C and lighting was set to mimic the natural photoperiod. Right before hatching we transferred embryos in groups of 60 (Bufo) or 30 (Rana) to containers with 5 L RSW to ensure constant density upon hatching.

Seven weeks before the start of the experiment, we placed 45-L plastic tubs (56 × 39 × 28 cm) in an open outdoor area and filled them with 40 L tap water. To each tub, we added 0.5 L pond water (containing phytoplankton and zooplankton) and 20 g dried beech (*Fagus sylvatica*) leaves to set up a self-sustaining ecosystem that provides shelter and nutrients for tadpoles. To prevent colonization by predators, we covered the tubs with mosquito net lids. Two days after hatching, we started the experiment by randomly selecting 44 healthy Bufo tadpoles and 24 Rana tadpoles from each family, and placing them into the tubs as follows. For each species, the nine families were divided into three groups of three families each, such that the first Bufo family group was paired up with the first Rana family group and so on. From each family group, we randomly distributed the tadpoles across the eight treatment groups (Figure 1a), with two replicates per family group × treatment combination, so there were six tubs in each treatment group (two from each family group). In total, we had 48 tubs arranged in six blocks, each block consisting of all treatments of a given family group. This design ensured that each tub contained siblings as well as non-kin tadpoles.

We terminated the experiment after 3 weeks because bufadienolide levels of Bufo tadpoles are highest and most sensitive to environmental conditions around the middle of larval development (Üveges et al., 2017). We weighed all tadpoles to the nearest 0.1 mg, and we preserved the Bufo tadpoles ($n = 398$) in HPLC-grade absolute methanol for chemical analysis of bufadienolides. We preserved the Rana tadpoles ($n = 216$) in 50% ethanol. We identified the developmental stage of all tadpoles according to Gosner (1960) by stereomicroscopic examination (we could not identify the developmental stage of one Rana tadpole because it was deformed).

All experimental procedures were carried out in accordance with Good Scientific Practice guidelines and national legislation. The Ethical Commission of the MTA ATK NÖVI approved the experiment, and the necessary permits were issued by the Government Agency of Pest County, Hungary (PE/KTF/3596-6/2016, PE/KTF/3596-7/2016 and PE/KTF/3596-8/2016).

2.3 | Chemical analysis

Each tadpole was homogenized and dried in vacuum to measure dry mass (±0.1 mg); then the samples were re-dissolved in 1 ml HPLC-grade absolute methanol and filtered using nylon syringe filters. Quantitative measurement of bufadienolide compounds was carried out by 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 analyser with electrospray ionization (ESI/MS). From each sample, 10 µL were injected and analysed at 35°C on a Kinetex C18 2.6 µm column (100 × 3 mm i.d.) in series with an octadecyl C18 guard column (4 × 3 mm i.d.). Eluent A was 5% aqueous acetonitrile with 0.05% formic acid and eluent B was acetonitrile with 0.05% formic acid. The flow rate was 0.6 ml/min and the gradient was as follows: 0–2 min, 10%–20% B; 2–15 min, 20%–32% B; 15–21 min, 32%–60% B; 21–21.5 min, 60%–100% B; 21.5–26 min 100% B; and 26–30 min 10% B. ESI conditions were 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; nebulizer N_2 gas flow, 1.5 L/min; positive ionization mode. Full scan spectra in the range of m/z (mass-to-charge ratio) values 350–800 were recorded, and selected-ion monitoring acquisition detecting the base peak of the bufadienolides we previously found in common toads (Bókony et al., 2016; Üveges et al., 2017) was performed as well. Bufadienolides were recognized by their characteristic UV spectrum, and identified by comparing their peak retention time and m/z to those of commercially purchased standards and to the peaks present in a toxin sample obtained from juvenile common toads (for more details, see Bókony et al., 2016; Üveges et al., 2017). The data were acquired and processed using LabSolutions 5.42v (Shimadzu).

We detected 24 bufadienolide compounds (Table S1). We used the calibration curve of the bufotalin standard to express the bufotalin-equivalent mass of each bufadienolide compound per sample (Benard & Fordyce, 2003; Hagman et al., 2009); then we summed the values of all compounds to estimate the total amount of bufadienolides per individual. This variable was then divided by tadpole dry mass to obtain the total amount of bufadienolides per body mass (mass-corrected amount of bufadienolides henceforward). We analysed both variables because they quantify two

different aspects of toxicity: the mass-corrected amount is more likely to express individual investment (i.e. proportion of resources allocated to toxin production) while the total amount is more likely to be relevant in inter-individual interactions (i.e. total toxin quantity available for allelopathy).

2.4 | Statistical analysis

All statistical analyses were run with R 3.3.1, using the packages "nlme" and "lsmeans". We used two alternative approaches as follows. First, we employed the concept of response surface analysis (Inouye, 2001) to assess how the tadpoles' mass, developmental stage and chemical defence varied with the density of both species. In these models, we assumed linear relationships, entering the number of Bufo and the number of Rana as covariates (numerical predictor variables) along with their interaction. Second, to be able to address potentially non-monotonous or cumulative effects of density, in another set of analyses we used the eight treatments as a fixed factor (categorical predictor variable). In these models, the proportion of variance explained by the treatments was tested using analysis of variance tables (i.e. *F*-tests) with type-III sums of squares; then, pairwise comparisons among treatment groups were tested by calculating linear contrasts and correcting the *p*-values for multiple testing with the FDR (false discovery rate) method (Pike, 2011).

All analyses were performed with linear mixed-effects (LME) models, in which we allowed for heteroscedasticity across treatment groups (Zuur, Ieno, Walker, Saveliev, & Smith, 2009) using the "varIdent" function in "lme" models. When the dependent variable was the total mass of tadpoles per tub, we used family group as a random factor. When the dependent variable was the body mass or developmental stage of individual tadpoles, number of bufadienolide compounds per tadpole, total or mass-corrected amount of bufadienolides, we used tub identity nested in family group as hierarchical random factors. We checked the requirements of LME analysis by inspecting residual plots; we $\log_{10}$-transformed the amount of bufadienolides (both total and mass-corrected) to improve the models' fit. All tests were two-tailed with 95% confidence level. Our analyses can be reproduced from Bókony, Üveges, Móricz, and Hettyey (2017).

3 | RESULTS

3.1 | Competitor biomass

The total mass of tadpoles per tub varied significantly among treatments ($F_{7,38} = 88.98$, $p < .001$, Figure 1b). The four high-density treatment groups did not differ among each other but had significantly larger total mass than the four treatment groups with medium or low density (Figure 1b). Also, the low-density group had significantly less total mass than two out of the three medium-density groups (Figure 1b). These differences agree well with our planned grouping of density treatments based on total mass (Figure 1a), except that total mass was smaller than we expected in tubs containing six Rana tadpoles (Figure 1b). This deviation from the planned densities arose because individual body mass did not differ significantly between the two species in the lower density treatments (Figure 1c), whereas at high densities Rana tadpoles had significantly (*c*. 1.5 times) larger body mass than Bufo tadpoles (Figure 1c).

3.2 | Effects on Bufo

The body mass of Bufo tadpoles was significantly reduced by high-density treatments ($F_{5,28} = 5.25$, $p = .002$; Figure 1c) and decreased with increasing numbers of both conspecific and heterospecific competitors (Table 1). The addition of one Rana was estimated to have about twice as large an effect as the addition of one Bufo (Table 1), suggesting that the effect of competitor biomass per species was similar; however, the effect of Rana was marginally non-significant, whereas the effect of conspecifics was highly significant (Table 1).

We detected 17–24 (most often 21–23) bufadienolide compounds in individual tadpoles (Table S1). While the number of compounds per tadpole showed a marginally non-significant tendency to increase with the number of conspecifics (Table 1), the number of Rana had no significant effect (Table 1) and none of the pairwise differences among treatment groups were significant after correction for multiple testing ($F_{5,28} = 2.15$, $p = .089$; Figure 1d).

In contrast, treatments had highly significant effects on the amount of bufadienolides (total amount per tadpole: $F_{5,28} = 4.24$, $p = .005$; mass-corrected amount: $F_{5,28} = 10.65$, $p < .001$). The total amount of bufadienolides per tadpole was not reduced at high density (Figure 1e), despite the smaller body mass of these tadpoles (Figure 1c). Instead, total bufadienolide amount was explained by a significant interaction between the numbers of Bufo and Rana tadpoles (Table 1, Figure S1): conspecifics had a significant, consistently positive effect while the effect of Rana was marginally non-significant and negative when they were few and increased as their numbers grew (Table 1, Figure S1). As a result, total bufadienolide amount was higher in the two treatments with the largest total mass containing 12 or 24 Bufo than in the three treatments containing six Bufo tadpoles irrespective of total mass (Figure 1e).

The mass-corrected amount of bufadienolides increased gradually with total competitor density (Figure 1f) and increased significantly with the number of conspecifics, whereas the number of Rana had no significant effect (Table 1). These differences in bufadienolide content were not attributable to developmental stage, because there was no significant variation in the developmental stage of Bufo tadpoles among treatment groups ($F_{5,28} = 1.20$, $p = .334$; Figure 2) and it was not significantly related to the number of conspecific or heterospecific competitors (Table 1).

3.3 | Effects on Rana

The individual body mass of Rana tadpoles did not vary significantly among treatment groups ($F_{4,23} = 0.56$, $p = .691$; Figure 1c) and was not significantly explained by the number of conspecific or heterospecific competitors (Table 1). Notably, the body mass of six Rana tadpoles was essentially the same when they were raised in the presence

TABLE 1 Results of response surface analysis testing the effects of Bufo and Rana tadpoles and their interaction

Dependent variable	Parameters[a]	Coefficient ± *SE*	*df*	*t*	*p*
Bufo tadpoles[b]					
Body mass (mg)	Intercept	300.957 ± 17.373	362	17.32	<.001
	Number of Bufo	−4.387 ± 0.992	30	−4.42	<.001
	Number of Rana	−8.764 ± 4.334	30	−2.02	.052
	Bufo × Rana	0.363 ± 0.488	30	0.74	.463
Developmental stage	Intercept	33.341 ± 0.434	362	76.83	<.001
	Number of Bufo	−0.011 ± 0.022	30	−0.50	.619
	Number of Rana	−0.091 ± 0.097	30	−0.93	.357
	Bufo × Rana	0.005 ± 0.011	30	0.47	.644
Number of bufadienolide compounds	Intercept	21.685 ± 0.428	362	50.64	<.001
	Number of Bufo	0.037 ± 0.019	30	1.98	.057
	Number of Rana	−0.017 ± 0.088	30	−0.19	.850
	Bufo × Rana	0.004 ± 0.009	30	0.40	.691
Total bufadienolide amount ($\log_{10}$ μg)	Intercept	0.644 ± 0.033	362	19.30	<.001
	Number of Bufo	0.005 ± 0.002	30	2.76	.010
	Number of Rana	−0.016 ± 0.008	30	−1.99	.056
	Bufo × Rana	0.002 ± 0.001	30	2.67	.012
Mass-corrected bufadienolide amount ($\log_{10}$ ng/mg)	Intercept	2.082 ± 0.053	362	39.37	<.001
	Number of Bufo	0.018 ± 0.003	30	6.76	<.001
	Number of Rana	0.010 ± 0.012	30	0.85	.403
	Bufo × Rana	0.001 ± 0.001	30	0.87	.392
Rana tadpoles					
Body mass[c] (mg)	Intercept	284.873 ± 52.567	186	5.42	<.001
	Number of Bufo	4.328 ± 8.958	24	0.48	.633
	Number of Rana	4.731 ± 5.177	24	0.91	.370
	Bufo × Rana	−0.867 ± 1.254	24	−0.69	.496
Developmental stage[d]	Intercept	28.768 ± 0.346	185	83.24	<.001
	Number of Bufo	−0.006 ± 0.061	24	−0.10	.919
	Number of Rana	0.047 ± 0.035	24	1.33	.195
	Bufo × Rana	0.002 ± 0.009	24	0.20	.840

[a]Parameters are given as the number of tadpoles per tub. To express the effect of Rana in biomass units (assuming that Rana grow twice as large as Bufo), divide the parameters "Number of Rana" and "Bufo × Rana" by 2.
[b]398 tadpoles in 36 tubs.
[c]216 tadpoles in 30 tubs.
[d]215 tadpoles in 30 tubs.

or absence of 12 Bufo tadpoles (Figure 1c). Developmental stage showed very limited variation among Rana tadpoles (Figure 2); it did not vary significantly among treatment groups ($F_{4,23}$ = 0.90, p = .479; Figure 2), nor with the number of conspecific or heterospecific competitors (Table 1).

4 | DISCUSSION

Our study yielded two main results. On the one hand, we found that Bufo tadpoles contained increased quantities of bufadienolides at higher competitor densities, demonstrating competition-induced plasticity in toxin production. On the other hand, we did not find support for the hypothesis that bufadienolides function to suppress heterospecific competitors, because the growth and development of Rana tadpoles was not inhibited by the presence of Bufo tadpoles and also because Rana tadpoles did not induce higher toxin production in Bufo tadpoles than conspecifics did.

To our knowledge, this is the first unequivocal evidence for induced toxin synthesis in response to increased competition in free-moving animals, demonstrating that phenotypic plasticity of chemical defence (or offence) is not limited to predator–prey interactions and immune responses in behaviourally and morphologically complex organisms (Hettyey et al., 2014; Tollrian & Harvell, 1999). This experimental result

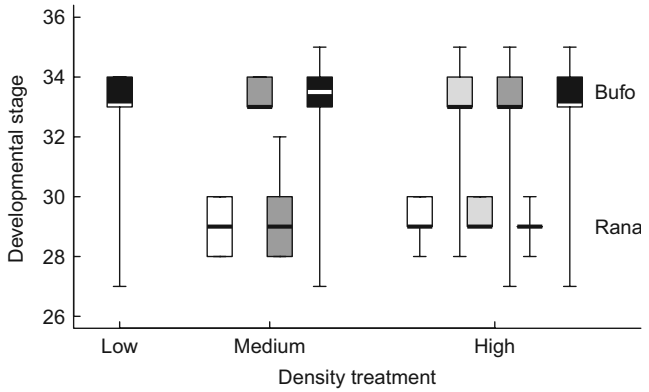

FIGURE 2 Developmental stage of Bufo (upper boxes) and Rana (lower boxes) tadpoles in the eight treatment groups. In each box plot, the thick middle line, box and whiskers represent the median, interquartile range and data range respectively. Box colour denotes the tubs' species composition as explained in Figure 1a (black: Bufo only, white: Rana only; dark grey: both species, more Bufo than Rana; light grey: both species, fewer Bufo than Rana)

corroborates our earlier finding that the toxin content of common toad tadpoles correlated positively with the density of competitors across natural ponds (Bókony et al., 2016). Such correlation may arise either by local adaptation in constitutive defences or *via* phenotypic plasticity (Bókony et al., 2016); our present results support the latter explanation. Furthermore, in another laboratory experiment, we found that the bufadienolide amount of common toad tadpoles increased when competition was simulated by decreasing food availability for small groups of tadpoles at a single density (Üveges et al., 2017). Although this might have been a stress response to hunger irrespective of competition, our present results clearly demonstrate that increased bufadienolide production is induced by competition even when food is relatively abundant (i.e. mortality was negligible). In both of our experiments, tadpoles reared in more competitive environments attained smaller body mass, but in spite of this inhibited growth, their total bufadienolide levels were at least as high or even higher compared to tadpoles reared in less competitive environments (Üveges et al., 2017; figure 1e,f in the present study). This suggests that competing tadpoles invested their resources into toxin production at the expense of growth; or alternatively, they may have been able to maintain or even increase their bufadienolide levels despite food limitation because the costs of bufadienolide synthesis may be low in terms of dietary resources (Kurali, Pásztor, Hettyey, & Tóth, 2016; Üveges et al., 2017). It is possible, however, that induced bufadienolide synthesis is traded off against long-term investment into critical life-history traits, as suggested by earlier studies (Benard & Fordyce, 2003; Hagman et al., 2009).

Although we found competition-induced changes in the bufadienolide content of Bufo tadpoles, the role of these chemicals in allelopathy remains unclear. We expected that bufadienolides would mainly be induced by, and effective against, heterospecific competitors because toxin-producing species should have evolved protection from autotoxicity; for example, consuming the bufadienolide-rich eggs or tissues of cane toads *Rhinella marina* has no ill effect on conspecific tadpoles but kills other species (Crossland & Shine, 2012; Crossland et al., 2011). However, in the common toad–agile frog system, we found no indication that interspecific competition would be the specific driver of toxin production. Bufo tadpoles' bufadienolide levels were not increased by the presence of Rana tadpoles more than by the same total mass of conspecific competitors, and the presence of Bufo larvae did not reduce the growth and development of Rana larvae. It is unlikely that the tadpoles could not discriminate between conspecific and heterospecific competitors (Relyea, 2002). Instead, a possible explanation for the lack of interspecific effects is that the encounter rate between the two species may have been relatively low, because Bufo larvae are more active and more gregarious than Rana larvae (our pers. obs.). If Bufo tadpoles use proximity or physical interaction (e.g. visual and tactile cues) for assessing competitor density (Rot-Nikcevic, Denver, & Wassersug, 2005) to adjust their toxin production, they will have perceived stronger competition by conspecifics than by Rana tadpoles. Low encounter rates might also explain the lack of allelopathic effects on Rana tadpoles, because bufadienolides are amphiphilic molecules so their highest concentrations are likely to occur at the interface of tadpole skin and water (Kubanek et al., 2002). In this case, allelopathy would become important only at very high interspecific encounter rates, e.g. when water depth is low due to desiccation (Cabrera-Guzmán, Crossland, & Shine, 2013), or at very low food availability which may increase the importance of scavenging on injured or dead toad tadpoles (Jefferson, Hobson, & Chivers, 2014; Jordan, Rombough, Pearl, & McCreary, 2004; Mahapatra, Dutta, & Sahoo, 2017; Wildy, Chivers, Kiesecker, & Blaustein, 2001).

Response surface analysis indicated that intraspecific competition had stronger effects on bufadienolide production than interspecific competition did, and high competitor biomass increased the total bufadienolide amount only when the majority of the competitors were conspecifics. This suggests that an important function of the inducibility of toxin production may be to mitigate some risk posed primarily by conspecifics; we propose two, mutually non-exclusive hypotheses. First, high densities and low per capita food levels are known to increase the incidence of intraspecific aggression and cannibalism in amphibian larvae (Jefferson et al., 2014; Jordan et al., 2004; Mahapatra et al., 2017; Wildy et al., 2001), and elevated bufadienolide levels might prevent or mitigate intraspecific biting by deterring conspecific attacks. Although toads are tolerant to the toxins of their own species (Crossland & Shine, 2011; Crossland et al., 2011), they still might find these substances distasteful as do many other species (Gunzburger & Travis, 2005). Alternatively, toad toxins may function in intraspecific chemical communication and species recognition (Crossland & Shine, 2011; Hagman & Shine, 2009), and thereby might help preventing cannibalistic attempts against kin in sibling schools which are characteristic of toad larvae (Blaustein, 1988).

The second possible function of competitor-induced chemical defence is the prevention of disease. Bufadienolides are known to have antimicrobial effects (Cunha Filho et al., 2005; Tempone et al., 2008), so they may be an important component of immune defence in toads which lack the antimicrobial skin peptides that are found in many other amphibians (Conlon, Iwamuro, & King, 2009). Infection risk can induce chemical defences, for example in leopard frog *Lithobates pipiens*

tadpoles, doubling the density of conspecifics caused more than 250% increase in skin peptides (Groner et al., 2014). Because the chances of transmitting parasites or pathogens are likely to be higher at high densities (Briggs, Knapp, & Vredenburg, 2010), and individuals are more likely to be susceptible to the diseases of conspecifics than other species (Freeland, 1983), our results are in concordance with the hypothesis that tadpoles produce more bufadienolides in response to elevated infection risk. It remains to be tested whether the upregulated bufadienolide production is effective in preventing disease transmission and/or cannibalistic interactions.

In sum, our results demonstrate that a form of chemical defence, considered to have evolved to provide protection against predators, can be induced by competitors. Although we found no indication of interspecific allelopathic effects, the potential of bufadienolides to mitigate infection risk and/or to prevent cannibalism makes them ideal candidates for multi-purpose allomones. So far, theoretical and empirical studies of inducible defences have, by far the most frequently, focused on the effects of predators (Tollrian & Harvell, 1999); the time is ripe for addressing the role of defensive and/or offensive chemicals against multiple enemies, and the consequences thereof for resource allocation trade-offs, life-history evolution and responses to anthropogenic change (Bókony, Mikó, et al., 2017; Hettyey et al., 2014).

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AUTHORS' CONTRIBUTIONS

A.H., B.Ü. and V.B. designed the experiment, B.Ü. performed the experiments, Á.M.M. performed the HPLC analyses; V.B. conducted the statistical analyses and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.q3g70 (Bókony, Üveges, et al., 2017).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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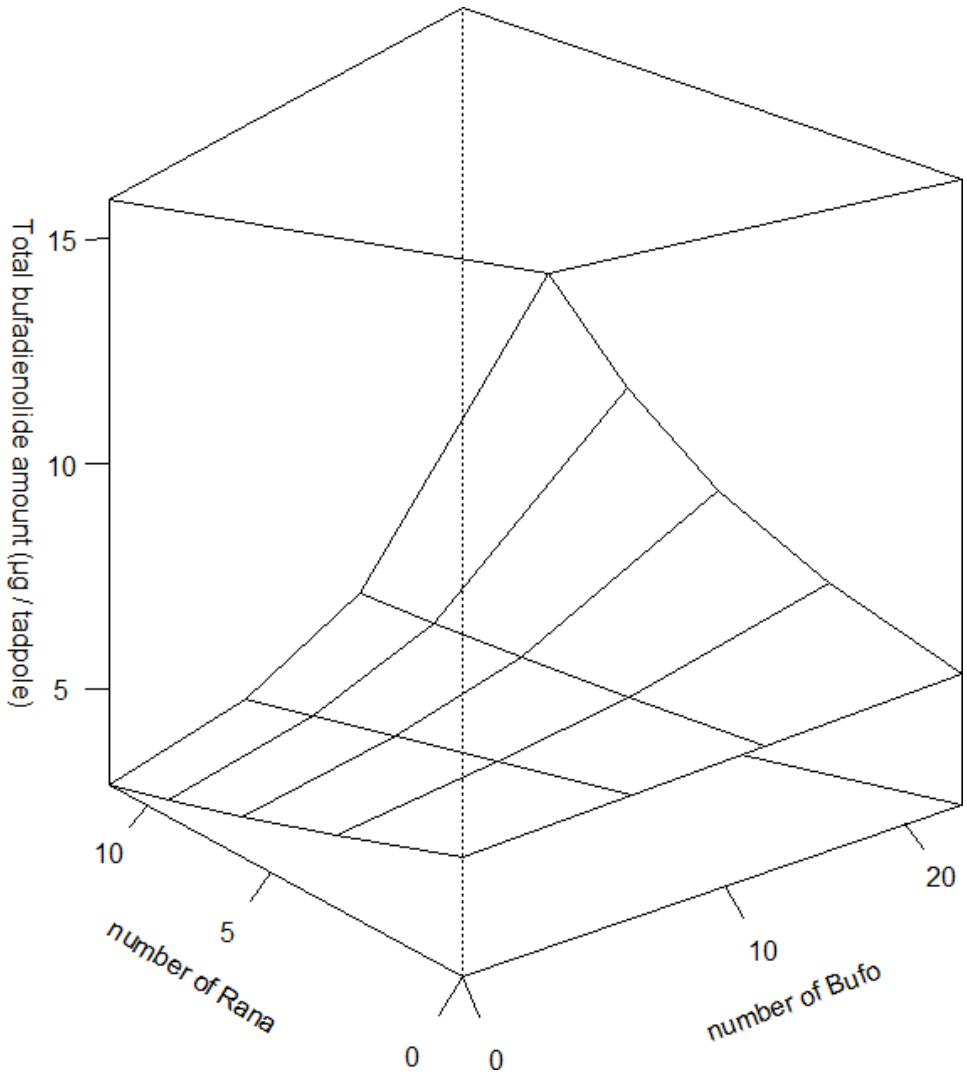
Supplementary Information for **Paper II**

Table S1. Bufadienolide compounds found in toad tadpoles, and the percentage of tadpoles in which each compound occurred.

Compound	Retention time (min)	Mass-to-charge ratio (m/z $[M+H]^+$)	Occurrence (%)
Bufotalin	10.8	445	35.2%
Telocinobufagin	9.7	403	60.6%
Unidentified compound 1	3.4	417	90.7%
Unidentified compound 2	4.1	419	95.0%
Unidentified compound 3	5.3	401	96.0%
Unidentified compound 4	5.5	415	100.0%
Unidentified compound 5	6.3	417	100.0%
Unidentified compound 6	6.4	615	89.9%
Unidentified compound 7	6.5	701	100.0%
Unidentified compound 8	6.7	717	100.0%
Unidentified compound 9	7.3	715	100.0%
Unidentified compound 10	7.4	731	100.0%
Unidentified compound 11	7.5	415	97.0%
Unidentified compound 12	8.1	729	100.0%
Unidentified compound 13	8.2	715	99.2%
Unidentified compound 14	9.0	727	100.0%
Unidentified compound 15	9.3	703	100.0%
Unidentified compound 16	10.2	729	100.0%
Unidentified compound 17	11.1	715	100.0%
Unidentified compound 18	11.6	401	77.1%
Unidentified compound 19	12.3	713	100.0%
Unidentified compound 20	14.8	757	100.0%
Unidentified compound 21	17.1	573	91.2%
Unidentified compound 22	18.4	571	94.2%

Figure S1. The fitted response surface of total bufadienolide amount per tadpole, showing the interaction between the number of Bufo and Rana tadpoles.



Paper III

Summary

Many organisms use inducible defences as protection against predators. In animals, inducible defences may manifest as changes in behaviour, morphology, physiology, or life history, and prey species can adjust their defensive responses based on the dangerousness of predators. Analogously, prey may also change the composition and quantity of defensive chemicals when they coexist with different predators, but such predator-induced plasticity in chemical defences remains elusive in vertebrates. In this study, we investigated if tadpoles of the common toad (*Bufo bufo*) adjust their chemical defences to predation threat in general and specifically to the presence of different predator species, furthermore we assessed the adaptive value of the induced defence. We reared tadpoles in presence or absence of four caged predator species in a mesocosm experiment, analysed the composition and quantity of their bufadienolide toxins, and exposed them to free-ranging predators. We show that toad tadpoles did not respond to predation risk by upregulating their bufadienolide synthesis. Fishes and newts consumed only a small percentage of toad tadpoles, suggesting that bufadienolides provided protection from vertebrate predators, irrespective of the rearing environment. Backswimmers consumed toad tadpoles regardless of treatment. Dragonfly larvae were the most voracious predators, but consumed fewer toad tadpoles if these were raised in the presence of dragonfly cues compared to their predator-naïve conspecifics. We propose that the presence of dragonfly larvae affected some unstudied aspect of anti-predator defence, which hindered the predator in feeding on toads. As an explanation to the lack of treatment effects on chemical defence, we propose, that the expression of predator-induced phenotypic plasticity may depend on other factors than immediate predation risk (e.g. local adaptations or presence of conspecifics), which may have obscured plastic chemical defence in our experiment.

Chemical defence of toad tadpoles under risk by four predator species

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Introduction

Phenotypic plasticity, the ability of a genotype to produce different phenotypes in response to varying environmental conditions (West-Eberhard, 1989; Futuyma, 1998; West-Eberhard, 2003), is a central topic of evolutionary ecology because of its fundamental role in shaping diversity, ecological processes (Miner *et al.*, 2005) and possibly even speciation (West-Eberhard, 1989; Agrawal, 2001; West-Eberhard, 2003; Pfennig *et al.*, 2010). Inducible defences are plastic responses evoked by predators and other enemies (Harvell, 1990; Tollrian & Harvell, 1999), which can affect predator-prey interactions and prey survival probabilities. For example, animals are capable of changing their behaviour, morphology, physiology, growth rate and development speed as a response to predation threat (West-Eberhard, 1989; Harvell, 1990; Tollrian & Harvell, 1999; Miner *et al.*, 2005).

Even though chemical defences are widespread among animals (Mebs, 2001; Brodie, 2009), and in many cases toxin compounds have been identified and their effects towards adversaries are well known (Blum, 1981; Tachibana, 1988; Pawlik, 1993; Toledo & Jared, 1995; Mebs, 2001; Savitzky *et al.*, 2012), they have been largely neglected in regard to phenotypic plasticity (Hettyey *et al.*, 2014). Only a few studies have tested for inducible chemical defences in animals, showing that sessile invertebrates do respond to predation risk by increased toxin production (Slattery *et al.*, 2001; Thornton & Kerr, 2002), and some vertebrates respond similarly to environmental stressors such as competitors and contaminants (Bókony *et al.*, 2017; Üveges *et al.*, 2017; Bókony *et al.*, 2018). Whether predators induce toxin synthesis in vertebrate prey has remained controversial (Benard & Fordyce, 2003; Hagman *et al.*, 2009; Bucciarelli *et al.*, 2017; Üveges *et al.*, 2017).

The density and composition of the predator fauna present in the environment can vary immensely and unpredictably over time and space, leading to considerable benefits of plastic adjustments in defensive traits, all the more so because different types of predators can differ in their dangerousness and in which defensive responses are effective against them. Accordingly, prey often respond to different predators with different changes in behaviour (Crowder *et al.*, 1997; Krupa & Sih, 1998; McIntosh & Peckarsky, 1999; Turner *et al.*, 1999; Relyea, 2003), morphology (Relyea, 2003; Kishida & Nishimura, 2005; Benard, 2006; Hoverman & Relyea, 2009) and life history (Relyea, 2003). Analogously, one can expect that prey individuals also adjust the composition or quantity of their defensive chemicals to the type of predators present, especially because predator species may also differ in their susceptibility to toxins (Gunzburger & Travis, 2005; Ujvari *et al.*, 2015; Mohammadi *et al.*, 2016). However,

to the best of our knowledge no study tested this hypothesis in toxin-producing vertebrates before.

Anuran amphibians are ideal model organisms for the study of phenotypic plasticity (Miner *et al.*, 2005). Changes in physiology, behaviour, morphology, and life-history traits of many anuran species have been shown to be inducible by predatory cues (Laurila *et al.*, 2002; Van Buskirk, 2002b; Kishida & Nishimura, 2005). Moreover, many anurans, including bufonid toads, rely on noxious skin secretions for protection against predators (Toledo & Jared, 1995; Gunzburger & Travis, 2005; Savitzky *et al.*, 2012). The main toxic compounds of the skin secretion of toads are cardiotoxic steroids called bufadienolides (Flier *et al.*, 1980; Toledo & Jared, 1995; Mebs *et al.*, 2007), which are synthesised by toads *de novo* (Chen & Osuch, 1969; Porto *et al.*, 1972; Üveges *et al.*, 2017) and are present in their tissues from a very early age on (Mebs *et al.*, 2007; Bókony *et al.*, 2016; Ujszegi *et al.*, 2017; Üveges *et al.*, 2017; Bókony *et al.*, 2018). These toxins are likely responsible for the fact that eggs, hatchlings and tadpoles of toad species are unpalatable to a wide variety of predators (Kruse & Stone, 1984; Henrikson, 1990; Denton & Beebee, 1991; Peterson & Blaustein, 1991; Toledo & Jared, 1995; Lawler & Hero, 1997).

Only a handful of studies tested so far if the bufadienolide synthesis of toads is inducible by environmental factors (Benard & Fordyce, 2003; Hagman *et al.*, 2009; Marion *et al.*, 2015; Bókony *et al.*, 2017; Üveges *et al.*, 2017; Bókony *et al.*, 2018), and provided inconclusive results, as they either found no evidence for predator-induced plasticity in chemical defence (Marion *et al.*, 2015; Üveges *et al.*, 2017), or the effect of predator cues presented during the tadpole stage could only be demonstrated after metamorphosis (Benard & Fordyce, 2003; Hagman *et al.*, 2009). Furthermore, only the study of Benard and Fordyce (2003) so far investigated whether inducible toxin production can enhance survival probability of toads when exposed to predators, but its results were equivocal because tadpoles of the American toad (*Anaxyrus boreas*) did not contain measurable amounts of bufadienolides and the effects observed in post-metamorphic toads were counterintuitive.

In this study we investigated whether tadpoles adjust their chemical defences to predation threat in general and specifically to the presence of four, phylogenetically distant predator species differing in voraciousness, and we further assessed the adaptive value of the induced defence. To accomplish these goals, we reared common toad (*Bufo bufo* (Linnaeus, 1758)) tadpoles in outdoor mesocosms in the presence or absence of caged predators, measured their bufadienolide content, and finally assessed their survival upon exposure to free-ranging predators. We chose the common toad as the study species, because its tadpoles display

relatively weak plastic responses to predators during the larval stage in terms of morphology and behaviour (Laurila *et al.*, 1998; Lardner, 2000; Van Buskirk, 2002a), but appear to be unpalatable to several predator species (Henrikson, 1990; Denton & Beebee, 1991), suggesting a heavy reliance on chemical defence. We predicted that tadpoles raised with caged predators would contain an elevated number of bufadienolide compounds and/or larger total bufadienolide quantity than their predator-naïve conspecifics. Also, we expected the strength of these responses to increase with predator dangerousness. Finally, we predicted that tadpoles exhibiting predator-induced phenotypes would have elevated survival probabilities compared to predator-naïve conspecifics when facing free-ranging predators. We collected further data on tadpole behaviour, body mass, morphology, length of larval development, and survival (for details see Appendix S1).

Materials and methods

Ethics statement

Permits for collection and transport of animals were issued by the City of Vienna (MA22–120657/2014) and by the Land Niederösterreich (RU5-BE-7/016-2014). Experimental procedures were approved by the institutional ethics committee and the national authority according to § 8ff of Law for Animal Experiments, Tierversuchsgesetz – TVG (GZ 68.205/0164-II/3b/2013).

Experimental procedures

We performed the experiment at the PNMS/PHS Sacré Coeur in Pressbaum, Austria, during spring 2014. We set up mesocosms ca. two weeks before the addition of toad eggs by filling plastic containers (82 × 58 × 30 cm, length × width × height) with 130 L tap water and adding 50 g dried beech (*Fagus sylvatica*) leaves to provide shelter for tadpoles and substrate for algal and microbial growth. Two days later we inoculated each mesocosm with 1 L pond water containing phyto- and zooplankton. To prevent colonization by predators we covered containers with mosquito nets. Mesocosms were arranged in a full-factorial randomised block design in which each block corresponded to one family of toads (see below). In each block, each experimental treatment was represented once (Fig. 1). Each mesocosm contained a cage in which we introduced a predator (except in the control treatment) one day before placing toad eggs into the mesocosms, as detailed below. Two further mesocosms per block containing an empty cage (i.e. no predator) served for raising additional predator-naïve tadpoles for the predation trials (as detailed below; Fig. 1).

We captured 12 amplexing pairs of common toads at Silbersee, Vienna, Austria (48°12'32.72"N, 16°15'47.61"E) and transported them to the site of the experiment. We allowed pairs to lay eggs in 45-L plastic containers placed outdoors, containing twigs as egg deposition substrates and filled with ca. 15 L aged tap water. On the day when the last pair finished egg deposition we randomly assigned ca. 120 developing eggs from each clutch to a given mesocosm and placed them into a plastic dish equipped with a mesh bottom floating on the water surface of each mesocosm. This way, already developing embryos were in contact with chemical cues present in the mesocosms. Captive pairs laid their eggs within 6 days, but developmental differences among clutches were not detectable upon hatching. Three weeks after egg laying, when tadpoles reached the free swimming state (developmental stage 20 according to Gosner, 1960), we haphazardly selected 60 healthy-looking individuals from each plastic dish and released them into the open water of the corresponding mesocosm (day 1 of the experiment). We removed remaining tadpoles and the plastic dishes from mesocosms.

To simulate predation threat, we collected 4th instar larvae of the southern hawker (*Aeshna cyanea* (Muller, 1764), hereafter dragonfly), adult backswimmers (*Notonecta* sp. Linnaeus, 1758), and adult male smooth newts (*Lissotriton vulgaris* (Linnaeus, 1758)) from private ponds in Austria, and acquired juvenile three-spined sticklebacks (*Gasterosteus aculeatus* Linnaeus, 1758) from a breeder. We obtained all predators before the start of the breeding season of common toads. Predators were housed in partially submerged cages, one cage per mesocosm, made from PVC tubes (21 × 11 cm, length × diameter), both ends covered with a double layer of mosquito netting. We fed each predator three times a week with one common toad and one common frog (*Rana temporaria* Linnaeus, 1758) tadpole (Table 1). We kept prey tadpoles of both species in separate containers that provided similar conditions for them as for focal tadpoles, but without the presence of any predator cues. We used common frogs as alternative prey to ensure that predators would provide all types and sufficient quantities of chemical cues indicating predation risk, even if predators do not consume all offered toad tadpoles (Table 1, Hettyey *et al.*, 2015). On each feeding occasion, we removed cages from the mesocosms, documented the number of surviving and consumed tadpoles since the last feeding event, replaced them with new ones and put the cages back into the water. To ensure uniform disturbance, we handled control cages in the same way, but without introducing tadpoles. When a predator died or did not eat for two consecutive feeding occasions, we replaced it with a new conspecific (substitute predators were kept in the same manner as specimens for the predation trials, see below). Survival of three predator species was high during the whole study: 20 out of 20 (100 %) dragonfly larvae, 14 out of 17 (82.35 %)

sticklebacks and 39 out of 40 (97.5 %) newts survived. However, out of 64 backswimmers only 14 (21.88 %) survived (varying total numbers arise from replacements of fasting or dead individuals).

To assess chemical defences of toad tadpoles, we collected samples on two occasions by conserving tadpoles in 70 % HPLC-grade methanol. First, we haphazardly selected one individual from each mesocosm thirteen or fourteen days after start of the experiment (developmental stage 29, $N = 60$; sampling lasted for two days because we also photographed tadpoles and measured their body mass, see Appendix S1). Second, we conserved the 10th toad tadpole to start metamorphosis (developmental stage 42) from each mesocosm ($N = 60$). Additional mesocosms that served to raise predator-naïve tadpoles for the predation trials (Fig. 1) were not sampled on either occasion.

After metamorphosis, surviving experimental animals entered another study (Üveges *et al.*, 2016). Adult toads, remaining tadpoles and predators, apart from sticklebacks, were released at their site of origin as soon as possible. Remaining sticklebacks were released into a private garden pond in Pressbaum, Austria.

Predation trials

We housed additional 24 specimens of each predator species separately during the study. We kept dragonfly larvae and backswimmers individually in 1 L (container size: 18 × 13 × 12 cm) and 3 L (29 × 19 × 14 cm) aged tap water, respectively; whereas sticklebacks and newts in groups of 12 in 40 and 20 L aged tap water, respectively (57 × 39 × 28 cm). Housing tubs of dragonfly larvae and backswimmers were equipped with a perching stick. We fed these predators three times a week *ad libitum* with *Tubifex* sp. (all predators), bloodworms (*Chironomus* sp.; dragonfly larvae), white mosquito larvae (*Chaoborus* sp.; backswimmers) and white worms (*Enchytraeus* sp.; sticklebacks and newts). To habituate predators to tadpoles, four and two days before the start of the predation trials (day ten and twelve) each predator received a toad and frog tadpole as prey. Predators received toad tadpoles at these two feeding occasions from the respective rearing container to which the given individual was *a priori* randomly assigned to.

To set up predation-trial venues, on day two of the main experiment we filled 45-L plastic tubs with 40 L aged tap water and added 0.3 L pond water and 9 g dried beech leaves to provide food and shelter for tadpoles. Eleven days later (day thirteen) we placed 6 toad tadpoles into each predation-trial tub, accompanied by 6 common frog tadpoles as alternative prey (Fig. 1). We introduced frog tadpoles to control for differences in voraciousness between individual

predators (since every predator received only toad tadpoles that were either raised with or without predatory-cues). Furthermore, predators are less discriminative and more likely to prey on chemically defended organisms when hungry than when satiated (Hileman *et al.*, 1994; Gillette *et al.*, 2000; Barnett *et al.*, 2007; Sandre *et al.*, 2010). Therefore, without the presence of alternative food source, i.e. frog tadpoles, predators may have had consumed highly defended toads (reared with predatory cues) at similar rates than poorly defended ones (controls), and thus the effect of hunger would have confounded our results. Toad tadpoles were haphazardly chosen from the experimental rearing tubs such that 6 tadpoles from one mesocosm were assigned to a predation-trial tub that would contain the same predator species they had been raised with (Fig. 1). Common frog tadpoles had no previous experience with predators. For each predator species and each toad family we used two predation-trial tubs: we introduced 6 toad tadpoles that had been raised with predators into one of the tubs, and we placed 6 predator-naïve control toad tadpoles into the other tub (Fig. 1). This resulted in 96 predation-trial tubs (4 predator species × 2 toad tadpole treatment, i.e. raised with or without a predator × 12 families).

Toad and frog tadpoles introduced into the predation trials were of somewhat different size (mean body mass ± SE; toads: 163.81 ± 2.04 mg, frogs: 121.81 ± 3.28 mg, based on subsamples of 58 individuals per species). Nonetheless, these size ranges correspond to relatively young tadpoles of intermediate sizes in both species, therefore it is unlikely that they posed a problem for even gape-limited predators (sticklebacks and newts; Semlitsch & Gibbons, 1988; Richards & Bull, 1990; Eklöv & Werner, 2000; Wilson & Franklin, 2000). After a 24-hour acclimatization period for tadpoles (on day fourteen) we released the assigned predator into each predation-trial tub. Predators were fasted for two days before the trial. Given that the four species of predators differ in voraciousness (Table 1), we determined the duration of the trials separately for each species (dragonfly larvae: 30 h, backswimmers: 48 h, sticklebacks: 84 h, newts: 120 h) by monitoring the predation-trial tubs and terminating all trials involving a given type of predator when approximately half of all the tadpoles were eaten. After termination we counted survivors of both tadpole species and assessed body size of predators by measuring wing length (dragonfly larvae), body length (backswimmers and sticklebacks) or snout-vent length (newts) to the nearest 0.01 mm using a digital calliper.

Analysis of toxin content

Preparation of samples and analysis of bufadienolide content of toads was carried out using high-performance liquid chromatography with diode-array detection and mass spectrometry

(HPLC-DAD-MS) according to already published protocols (Bókony *et al.*, 2016; Bókony *et al.*, 2017; Üveges *et al.*, 2017; Bókony *et al.*, 2018).

Statistical analyses

Toxin content of tadpoles was assessed using three variables: number of bufadienolide compounds (NBC), total bufadienolide quantity (TBQ), and mass-corrected total bufadienolide quantity (mcTBQ). When determining NBC for each animal, we considered a compound to be present when its signal to noise ratio was at least 3 in the chromatogram. We estimated the quantity of each compound from the area values of chromatogram peaks based on the calibration curve of the bufotalin standard and summed up these values to obtain estimates of TBQ for each individual. This approach yields approximate estimates of bufadienolide quantities, and has been used in similar studies (Benard & Fordyce, 2003; Hagman *et al.*, 2009; Bókony *et al.*, 2016; Üveges *et al.*, 2017; Bókony *et al.*, 2018). We calculated mcTBQ by dividing TBQ by the dry mass of individuals. TBQ measures the total toxin content of a whole tadpole, relevant for anti-predatory defence, whereas mcTBQ represents a proxy of the relative amount of resources allocated into toxin synthesis.

We analysed the effects of predator treatment on toxin content using linear mixed-effects models (LMM). We entered NBC, TBQ or mcTBQ as the dependent variable into separate models. In case of NBC and TBQ, initial models included treatment and age of tadpoles (developmental stage 29 or 42) as fixed factors, dry mass as a covariate, and all two-way interactions and the three-way interaction. In case of mcTBQ the initial model included treatment and age as fixed factors and their two-way interaction. In all models, mesocosm nested within family were included as random factors. We applied stepwise backward model-simplification based on *p*-values with $\alpha = 0.05$ (Grafen & Hails, 2002). We present final models containing significant terms (for relevant statistics on non-significant terms see Table S1 and Table S2). We ran all analyses in R 3.4.0 (R Development Core Team, 2017) using the 'lme' function in the 'nlme' package (Pinheiro *et al.*, 2017). *P*-values were calculated with the 'Anova' function in the 'car' package (Fox & Weisberg, 2011), using type-2 sums of squares (as suggested by Langsrud, 2003 and Hector *et al.*, 2010 for models with interactions). Two samples were discarded from these analyses, because their dry mass was measured incorrectly (see Appendix S2). Additionally, we described the within-individual diversity of bufadienolide compounds by applying hierarchical diversity partitioning using the 'hierDiversity' package (Marion *et al.*, 2015); for further information on this approach see the Appendix S1.

We analysed survival of toad tadpoles in the predation trials for each predator species separately using generalized estimation equations (GEE) models with binomial distribution (we chose this approach because the effect of predator size could not be modelled adequately using LMM with these data; Zuur *et al.*, 2009). As the dependent variable we entered the proportion of toad tadpoles surviving out of all toad tadpoles in the predation-trial tub. Initial models included toad tadpole treatment (i.e. predator-naïve or raised with caged predator) as a fixed factor, and the number of frog tadpoles eaten during the predation trial and predator size (to control for potentially different voraciousness between predators) as covariates. All models included toad family as the random factor. We ran analyses using the 'geeglm' function in the R package 'geepack' (Venables & Ripley, 2002). We performed model simplification as described in the case of toxin content, but since there were no factors with more than two categories in these models, we evaluated the *p*-values using the 'summary' function in 'geepack'. Confidence intervals for the survival estimates in the two treatment groups were calculated from linear contrasts of the final models using the 'lsmeans' function in the 'lsmeans' package (Lenth, 2016). Only one newt ate a toad tadpole, therefore we did not perform a formal analysis of survival in the presence of free-ranging newts. Further, two backswimmers, one dragonfly, and one stickleback did not consume any tadpoles (neither toads nor frogs, see Appendix S2). Consequently, we could analyse survival in the remaining 22 trials involving backswimmers, 23 trials involving dragonfly larvae and 23 trials involving sticklebacks.

Results

Antipredator responses in toxin content

Predator treatments had no significant effect on total bufadienolide quantity (Table 2, Fig. 2, Table S1) or on the within-individual diversity of bufadienolides (Fig. S1). However, the interaction of tadpole dry mass and predator treatment had a significant effect on the number of bufadienolide compounds (Table 2): heavier tadpoles raised in the presence of sticklebacks had fewer bufadienolide compounds than expected from the allometric relationship between dry mass and NBC of control tadpoles (Table S2, Fig. S2). The other three predator species had no significant effect on NBC (Table S2, Fig. 2). Furthermore, compared to individuals that started metamorphosis, tadpoles had on average 39.36 % (mean ± SE of difference: 5.02 ± 0.23) more bufadienolide compounds, 15.54 % (340.83 ± 115.07 ng) higher TBQ and 42.26 % (75.75 ± 9.32 ng/mg) higher mcTBQ (Fig. 2).

Other phenotypic responses to predators

We found no significant effect of predator treatment on survival, behaviour, body mass or morphology of toad tadpoles (see Appendix S1). Time to metamorphosis was significantly shorter in the presence of sticklebacks than in control tubs (Fig. S3), whereas the other three predators did not affect length of larval development (see Appendix S1).

Predation trials

When exposed to free-ranging dragonfly larvae, toad tadpoles that developed in the presence of caged specimens of this predator had on average 25.1 % higher survival compared to their predator-naïve conspecifics (Table 3, Fig. 3, Table S3). The presence of caged backswimmers, sticklebacks and newts during tadpole development did not have a significant effect on toad tadpole survival in predation trials (Table 3, Fig. 3, Table S3).

Discussion

We found no evidence that common toad tadpoles respond to the presence of four different predator species by upregulating their bufadienolide synthesis. This finding may be explained in several ways. First, bufadienolide production may be non-inducible in general. However, previous results on common toads and related species dismiss this explanation by demonstrating plastic adjustment of bufadienolide production in response to a variety of environmental factors, such as restricted food levels (Üveges *et al.*, 2017), a herbicide (Bókony *et al.*, 2017) and competitors (Bókony *et al.*, 2018). Second, larval bufadienolide synthesis may not be inducible by predators specifically, since no studies to date have found plastic changes in bufadienolide content of tadpoles in response to predator cues (Benard & Fordyce, 2003; Üveges *et al.*, 2017). However, when toad tadpoles were raised with predator cues, differences in chemical defences between control and predator-exposed individuals became apparent after metamorphosis (Benard & Fordyce, 2003; Hagman *et al.*, 2009), which suggests that toads respond to larval predation risk by some physiological changes in the bufadienolide synthesis pathway or anatomical changes in toxin-producing structures that become detectable only during or after metamorphosis. A third explanation suggests that predator-induced phenotypic plasticity does exist in bufadienolide production of toad tadpoles, but its expression depends on factors other than immediate predation risk, and this context-dependence can hinder the detection of predator-induced plastic changes in chemical defences.

We propose two factors that could have obscured plastic chemical defences in our experiment: habitat of origin and presence of conspecifics. Through genetic assimilation (West-

Eberhard, 2003; Crispo, 2007; Pfennig *et al.*, 2010) an originally plastic trait can become fixed in environments where a relevant inducing biotic or abiotic factor is persistent. Tadpoles used in the current study originated from a permanent pond inhabited by fishes. Because fishes have persisted for many generations in this aquatic habitat, and they are one of the most voracious predators of amphibian larvae (Wells, 2007), it is possible that selection acted to reduce plasticity in bufadienolide synthesis in this population. To what extent genetic assimilation may have influenced our results, and may in general lead to among-population variation in plasticity of chemical defences remains unknown.

Another environmental factor which may influence toxin production and may have obscured plastic antipredator responses in our study is the presence of competitors. A recent study showed that increased conspecific density can induce elevated bufadienolide synthesis in toad tadpoles (Bókony *et al.*, 2018). Because in the present study tadpoles were reared at relatively high densities (approx. 60 tadpoles at the beginning of the experiment and 35 tadpoles after day thirteen-fourteen in 130 L water), it is possible that competitors induced intensive bufadienolide production regardless of the presence or absence of predators, so that a further increase in toxin content in response to predators was either not necessary or physiologically not possible. Further experiments are needed to explicitly test this idea.

We found that dragonflies posed the biggest threat to toad tadpoles, followed by backswimmers, sticklebacks and newts in this order (Table 1, Fig 3.). The predation trials revealed that tadpoles raised with dragonfly larvae survived better, compared to predator-naïve tadpoles, when they were exposed to this predator. Because we could not detect any significant phenotypic responses induced by the presence of caged dragonflies during tadpole development, we speculate that this treatment affected some unstudied aspect of behaviour, morphology, physiology or chemical defence of tadpoles (e.g., enhanced schooling behaviour or elevated synthesis of non-bufadienolide defensive chemicals) that provided an effective defence against this predator. We did not observe differences in survival in predation trials between control tadpoles and their siblings raised with backswimmers, newts or sticklebacks, similarly to earlier findings with various predators (McCollum & Van Buskirk, 1996; Van Buskirk & Relyea, 1998). However, when confronted with these predators, especially the vertebrate species, survival of toad tadpoles was very high (Fig. 3), leaving little variation for an effect of the rearing environment to manifest in. Similarly, during feeding sessions in the rearing stage of the current experiment, newts and sticklebacks consumed fewer of the offered naïve toad tadpoles than did backswimmers and dragonflies (Table 1). Also, for newts and sticklebacks, toad tadpoles were significantly less palatable than common frog larvae, as

demonstrated by the results of our feeding sessions in the rearing stage (Table 1) as well as the predation trials (Fig. S4) irrespective of whether or not the toad tadpoles had been raised with predators. This suggests that the "baseline" toxin levels in the studied toad population are high enough to provide effective defence against newts and fish.

This differential susceptibility of toad tadpoles to invertebrate and vertebrate predators is consistent with earlier results: typically, invertebrates find chemically defended tadpoles more palatable than do vertebrates (Gunzburger & Travis, 2005). This difference may, at least partly, be due to disparate sensitivity to bufadienolides, which inhibit Na^+/K^+ ATPases through attaching to the oubain binding site of these enzymes (Flier *et al.*, 1980; Pierre & Xie, 2006; Schoner & Scheiner-Bobis, 2007; Lingrel, 2010). Indeed, some species find bufadienolide-containing prey unpalatable (Kruse & Stone, 1984; Henrikson, 1990; Denton & Beebee, 1991; Peterson & Blaustein, 1991; Toledo & Jared, 1995; Lawler & Hero, 1997), while others appear to be resistant to these compounds (Dobler *et al.*, 2012; Ujvari *et al.*, 2015; Mohammadi *et al.*, 2016; Arbuckle *et al.*, 2017), some of which, interestingly, are not known to be specialized predators of bufadienolide-containing prey (Mohammadi *et al.*, 2016). The high palatability of toad tadpoles to dragonfly larvae might be due to such a resistance. Furthermore, utilizing a special feeding apparatus may also circumvent chemical defences of toad tadpoles: the pierce and suck feeding method of backswimmers may allow them to avoid the ingestion of bufadienolides produced and stored mainly in the skin of toads (Toledo & Jared, 1995; Halliday *et al.*, 2009). On the other hand, species that engulf their entire prey and do not seem to have evolved resistance against bufadienolides, such as smooth newts and sticklebacks, likely become fully exposed to the toxic effects of tadpoles' chemical defences upon ingestion.

We are highly confident that the lack of significant treatment effects in our experiment is not due to methodological shortcomings. A large number of studies using very similar methodology produced reliable results on inducible defences in larval anuran amphibians (e.g. Van Buskirk, 2009; Hettyey *et al.*, 2011, for a review see Wells, 2007). Also, previous studies exposing toad tadpoles specifically, to very similar conditions reported plastic phenotypic responses even when concentrations of chemical cues from predators were more diluted than what we applied (in our experiment: one dragonfly / 0.48 m^2 in 130 L water versus two crayfish and/or 1 trout / 2.6 m^2 in 1000 L water, Nyström & Åbjörnsson, 2000; or 2.2 dragonfly larvae / m^2 in 560 L water, Van Buskirk, 2002a). Furthermore, we found that in the presence of sticklebacks, toad tadpoles metamorphosed earlier compared to control animals (Fig. S3), which suggests that tadpoles perceived fish cues and reacted by enhancing allocation into development presumably to leave the dangerous waters as soon as possible (Laurila *et al.*, 1998;

Chivers *et al.*, 1999). We also found that the largest tadpoles raised in the presence of fish cues produced a lower number of bufadienolides at metamorphosis than expected. It is possible that such tadpoles maximized growth at the expense of bufadienolide synthesis to reach a size refuge against sticklebacks (Semlitsch & Gibbons, 1988; Richards & Bull, 1990; Eklöv & Werner, 2000). Finally, tadpoles raised in the presence of dragonfly larvae enjoyed an enhanced survival probability as compared to their predator-naïve sibs. These treatment-dependent effects together suggest that tadpoles did perceive the presence of predators during their development and were able to respond to them phenotypically, therefore the lack of responses in chemical defences was not due to an inability of tadpoles to sense olfactory cues on predation risk. On the other hand we also do not suspect the lack of significant treatment effects to be an artefact of the insensitivity of the chemical analytical framework, since the same build has proven to be effective in providing evidence for inducible bufadienolide synthesis in the same study species in the past (Bókony *et al.*, 2017; Üveges *et al.*, 2017; Bókony *et al.*, 2018).

Taken together, we did not find signs of inducible antipredator responses in the chemical defences of toad tadpoles originating from a population that coexists with predatory fishes. The observed level of chemical defence apparently provides protection from several vertebrate predators, while it defends less efficiently against invertebrates, which seem to be better able to cope with toad toxins. These results suggest that toad tadpoles currently may have the upper hand in the evolutionary arms race against some, but not all aquatic predators. Generally, the current study, with the addition of previous results emphasizes that vertebrate chemical defences may be influenced by a complex array of factors, including the evolutionary past of predator-prey coexistence, the predators' susceptibility to toxins, and prey's exposure to non-predatory environmental stressors (Bókony *et al.*, 2016; Bókony *et al.*, 2017; Üveges *et al.*, 2017; Bókony *et al.*, 2018); therefore the detection of inducible chemical defences requires comprehensive understanding of this complexity.

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Supporting information

Appendix S1: Additional methods and results as well as tables and figures.

Appendix S2: Datasets for the analysis of toxin content of toad tadpoles and survival in the predation trials. Available in the figshare repository (https://figshare.com/s/4a35e739e62fc3cc814d, DOI: 10.6084/m9.figshare.5777550).

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Table 1: Percentage of tadpoles consumed by predators over the feeding sessions during the rearing period of the experiment. Mean ± SE and range (in brackets), as well as the results of generalized linear model with quasibinomial distribution comparing the survival of toad and frog tadpoles are presented. *P* values were calculated using the 'car' package in R using type-2 sum of squares.

Predator species	% toad larvae	% frog larvae	χ^2	df	*p*
dragonfly larvae	91.18 ± 2.41 (80.77 - 100)	93.71 ± 2.39 (84.62 - 100)	1.1	1	0.294
backswimmer	73.66 ± 3.05 (62.96 - 88)	89.94 ± 3.18 (74.07 - 100)	23.36	1	<0.001
stickleback	32.51 ± 6.33 (10.34 - 60.71)	94.5 ± 2.83 (80.77 - 100)	109.93	1	<0.001
smooth newt	6.53 ± 1.75 (0 - 15.38)	72.77 ± 4.4 (57.69 - 89.29)	279.99	1	<0.001

Table 2: Effects of age, dry mass, predator treatment and their interaction on bufadienolide toxin content of common toad tadpoles. We present terms included in the final LMM models; type-2 sums of squares were calculated with the 'Anova' function in the 'car' package. Statistics for the initial, full models (including terms removed during model simplification) are presented in Table S1.

	χ^2	df	*p*
Number of bufadienolide compounds (NBC)			
age	484.73	1	<0.001
dry mass	7.19	1	0.007
treatment	2.07	4	0.723
dry mass × treatment	10.50	4	0.033
Total bufadienolide quantity (TBQ)			
age	8.77	1	0.003
Mass-corrected total bufadienolide quantity (mcTBQ)			
age	66.07	1	<0.001

Table 3: Effects of predator treatment on survival of toad tadpoles in the predation trials. We present parameter estimates from GEE models; the 'intercept' shows the logit of survival for the control tadpoles and the 'treatment' parameter shows the difference in logit survival between the tadpoles raised with the respective predator and the control tadpoles. Note that predator size and the number of common frog tadpoles eaten had non-significant effects; statistics for the initial, full models are presented in Table S3. We did not analyse predation trials involving newts because overall only one of these animals consumed a toad tadpole.

	N	Estimate	SE	Wald χ^2	*P*
dragonflies	23				
intercept		-1.335	0.318	17.60	<0.001
treatment		1.169	0.323	13.10	<0.001
backswimmers	22				
intercept		1.105	0.469	5.54	0.019
treatment		0.045	0.405	0.01	0.912
sticklebacks	23				
intercept		1.849	0.600	9.50	0.002
treatment		0.097	0.874	0.01	0.911

Fig. 1: Schematic diagram of the experimental design, showing the experimental units on the example of one hypothetical common toad family. Upper, middle and lower units represent mesocosms of toad tadpoles, predation-trial tubs and mesocosms of naïve frog tadpoles, respectively. Abbreviations represent predator treatments as follows: D: dragonfly larva, B: backswimmer, S: stickleback, N: newt, C: control. Each microcentrifuge tube represents two toads sampled for toxin analysis (one during the tadpole stage and one at the start of metamorphosis).

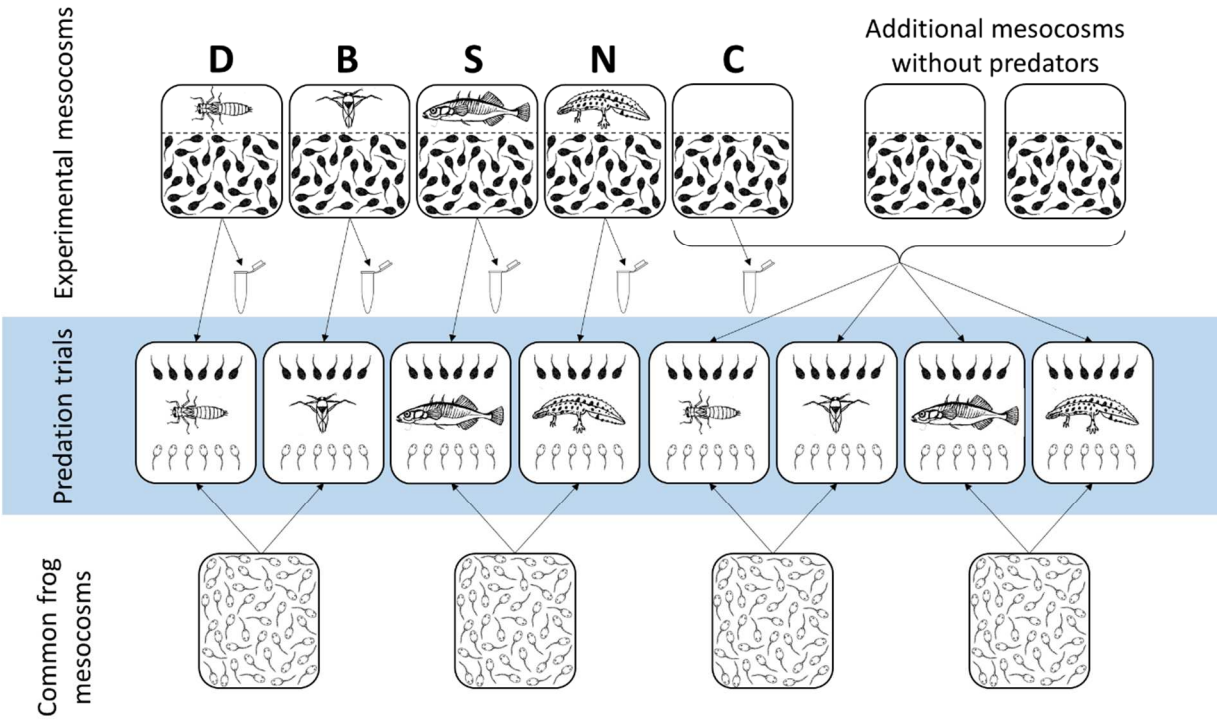


Fig. 2: Toxin content of toads ca. midway through larval development (developmental stage 29) and at the onset of metamorphosis (developmental stage 42). **A:** Number of bufadienolide compounds (NBC). **B:** total bufadienolide quantity (TBQ). **C:** mass-corrected total bufadienolide quantity (mcTBQ). Thick horizontal lines and boxes represent the medians and interquartile ranges, respectively; whiskers extend to the upper and lower quartile ± 1.5 × interquartile range; open circles represent extreme data points.

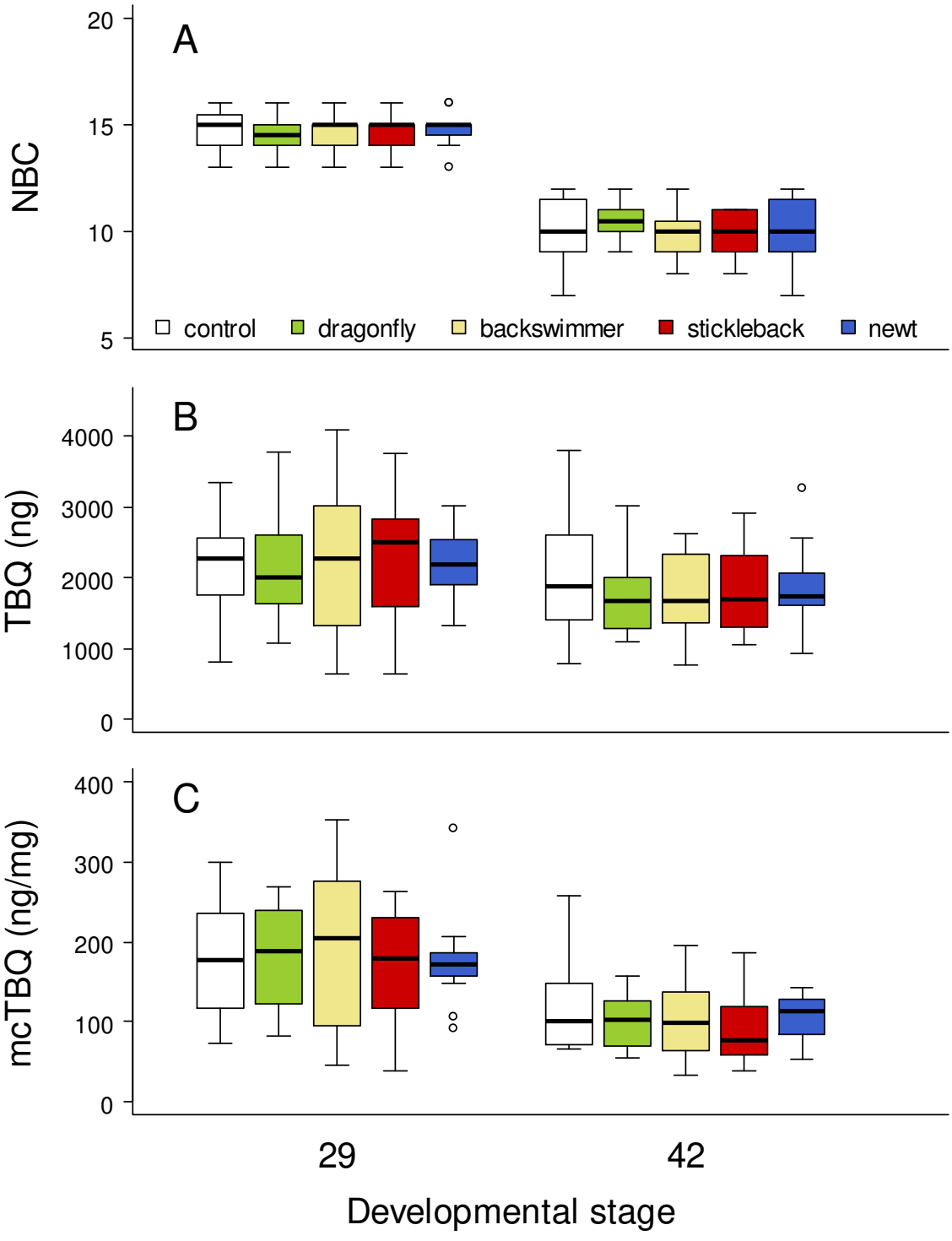
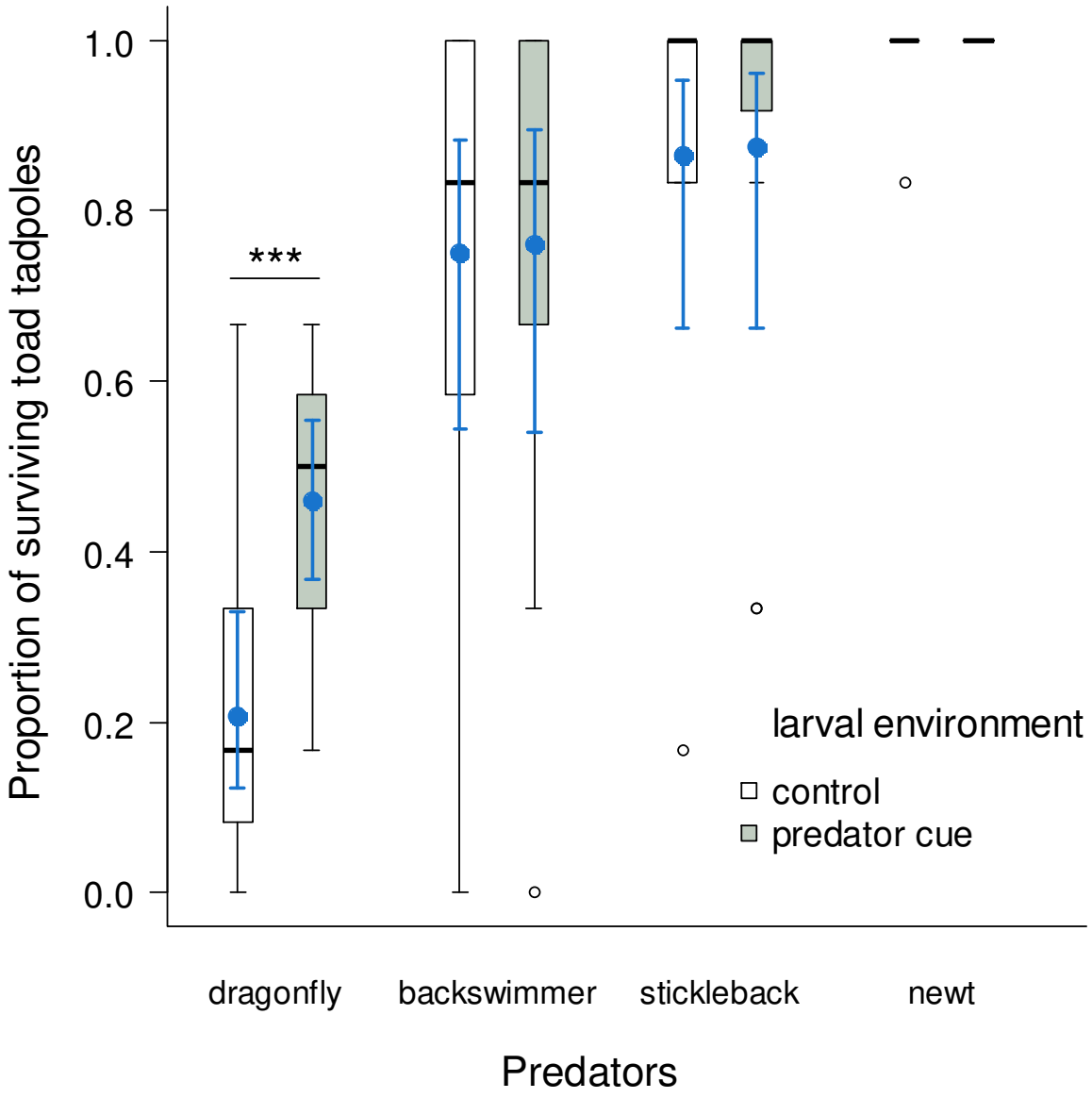


Fig. 3: Proportion of surviving toad tadpoles in the predation trials. A significant difference is marked with asterisks ($P < 0.001$). For the interpretation of box plots see Fig. 2. Filled circles and error bars represent means ± 95% confidence intervals calculated from GEE models.



Supplementary Information for **Paper III**

Additional methods and results

Here follows a brief summary of additional methods and results. Further details are available upon request from the authors.

Diversity of bufadienolide compounds

We quantified the diversity of bufadienolide compounds by applying hierarchical diversity partitioning using the 'hierDiversity' package in R (Marion *et al.*, 2015). Hierarchical diversity partitioning uses the concept of diversity indices, routinely applied in community ecology, to describe complex phenotypes (i.e. the whole poison secretion of toad tadpoles) as the diversity (effective number) of underlying phenotypical components (i.e. the individual bufadienolide compounds in the poison mixture). Diversity is partitioned into an intra-individual (α) and an inter-individual (β) component. In our case, α is interpreted as the diversity of individual bufadienolide compounds of tadpoles, whereas β represents the diversity of distinct toxin profiles (i.e. bufadienolide "cocktails" found within individual tadpoles) among our samples. Using this approach, we found that the predator treatments did not affect the diversity of bufadienolide compounds in our study (Fig. S2).

Behaviour

We observed behaviour of toad tadpoles on days eleven, twelve and thirteen of the experiment. At the beginning of each day the mosquito netting was removed from the mesocosms, and two observers each counted the number of visible tadpoles and the number of active visible tadpoles in the mesocosms four or five times a day for the first two days, and three times on the third day. In total we obtained 25 observations of behaviour for each mesocosm.

At each observation we registered the number of tadpoles that were visible, active and in the third of the mesocosm where the predator cage was located. For the analysis we calculated daily mean percentage values of these numbers (N = 180 mesocosm observations): we used the percentage of total number of tadpoles that were visible (visibility), the percentage of visible tadpoles that were active (activity) and the percentage of visible tadpoles in the third of the mesocosm where the predator cage was located (position). To calculate visibility we used the initial total number of tadpoles in each tub because overall survival was high in our experiment and treatment had no significant effect on survival (see section *Survival*). We analysed the obtained percentages using linear mixed-effects models (LMM). Initial models included

visibility, activity or position as the dependent variable, predator treatment as a fixed factor and family as random factor. We also included another random term in the models, with date as the random slope and mesocosm as the random intercept. We ran analyses in R 3.4.0 (R Development Core Team, 2017) using the 'lmer' function in the 'lme4' package (Bates *et al.*, 2015). *P*-values were calculated with the 'anova' function in 'lmerTest' (Kuznetsova *et al.*, 2017), using the Satterthwaite approximation for degrees of freedom. These analyses showed that neither the visibility ($F_{4,44}$ =1.046, p = 0.395), activity ($F_{4,45.44}$ =1.376, p = 0.257), nor the position ($F_{4,55}$ =0.954, p = 0.440) of tadpoles was significantly affected by predator treatment.

Body mass

On day thirteen or fourteen of the experiment we haphazardly removed 8 toad tadpoles (developmental stage 29) from each mesocosm. After anesthetising them with 0.05 w/w% MS-222 (tricaine-methanesulfonate, Sigma-Aldrich) we photographed them (see section *Morphology* below) and measured their body mass using an analytical balance to the nearest mg (N = 480 tadpoles). These animals were released at their pond of origin after the measurements were taken. Subsequently, we also measured the body mass of all animals remaining in the mesocosms the same way on the day they started metamorphosis (developmental stage 42, N = 1978 metamorphs).

We analysed body mass as the dependent variable in LMMs, using the 'lme' function in the R package 'nlme' (Pinheiro *et al.*, 2017). In the model with tadpoles we entered predator treatment as fixed factor and mesocosm nested in family as random factors. In the model with metamorphs we also entered length of larval development (number of days from the start of the experiment to reaching developmental stage 42) and the number of surviving conspecifics in the same mesocosm as covariates. These analyses showed that body mass of neither tadpoles (treatment: $F_{4,44}$ =1.736, p = 0.159), nor metamorphs (full model: treatment: $F_{4,43}$ =1.125, p = 0.304; development time: $F_{1,1917}$ =277.118, p = <0.001; number of conspecifics: $F_{1,43}$ =1.814, p = 0.185; no variable became significant during model simplification) was affected significantly by predator treatment.

Morphology

After anesthetising them, we placed tadpoles into a Plexiglas chamber filled with aged tap water. The chamber was part of a device that allowed us to take simultaneous photos from both the side and ventral views. From the photos we obtained the following body shape variables

using ImageJ 1.46r (Schneider *et al.*, 2012): body length, body depth, body width, tail length, tail depth, tail muscle depth and tail muscle width.

We regressed each of the above body-shape variables against the square-root of body mass (transformation was applied to normalize the distribution of data) and used the mesocosm mean of these residuals for further analysis (N = 60 mesocosms). We analysed morphology using a multivariate general linear model in IBM SPSS Statistics 20.0 (SPSS Inc., Chicago, USA), including the mesocosm mean of body shape residuals as dependent variables, and predator treatment and family as fixed factors. We found that predator treatment had no significant effect on the morphology of toad tadpoles (Wilk's $\lambda = 0.743$, $F_{24, 137.265} = 0.509$, $p = 0.972$).

Length of larval development

We analysed development time (the median value of days needed to reach developmental stage 42 among all individuals in a mesocosm, N = 60 mesocosms) as the dependent variable, predator treatment as fixed factor and family as random factor in a LMM. We used the 'lme' function in the R package 'nlme' (Pinheiro *et al.*, 2017) for analysis. Pairwise comparisons among treatment groups were tested by calculating linear contrasts corrected for false discovery rate (Benjamini & Hochberg, 1995) in the 'lsmeans' package (Lenth, 2016) in R.

This analysis showed that predator treatment had a significant effect on the development time of toads ($F_{1,44} = 3.355$, $p = 0.018$): tadpoles that developed in the presence of sticklebacks metamorphosed on average 1.13 ± 0.345 days (mean ± SE) earlier than control animals (t = -3.265, df = 44, $p = 0.009$; Fig. S3). Backswimmers (t = -1.572, df = 44, $p = 0.246$), dragonfly larvae (t = -0.605, df = 44, $p = 0.631$) and newts (t = -0.484, df = 44, $p = 0.631$) did not have a significant effect on development time (Fig. S3).

Survival

We analysed survival of toad tadpoles using a generalized linear mixed-effects model with quasi-binomial error distribution, using the 'glmmPQL' function in the 'MASS' package (Venables & Ripley, 2002). We entered the proportion of individuals surviving to developmental stage 42 (N = 60 mesocosms) as the dependent variable, predator treatment as fixed factor, development time (the mesocosm median of days needed to reach developmental stage 42) as covariate, and the treatment × development time interaction; and we added family as random factor. P-values were calculated with the "Anova" function in the "car" package (Fox & Weisberg, 2011), using type-2 sum of squares.

Survival of toad tadpoles was overall very high in our study (mean: 94.29 %, 95% confidence interval: 89.86 – 98.94 %), and neither predator treatment (χ^2 = 2.70, df = 4, p = 0.610), nor development time (χ^2 = 0.33, df = 1, p = 0.567) had a significant effect. The treatment × development time interaction was also non-significant (χ^2 = 4.57, df = 4, p = 0.334).

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Tables

Table S1: Effects of age, dry mass, predator treatment, and their interactions on bufadienolide toxin content of common toad tadpoles. We present all terms included in the full LMM models; type-2 sums of squares were calculated with the 'Anova' function in the 'car' package. Significant terms are highlighted in bold.

	χ^2	df	*p*
Number of bufadienolide compounds (NBC)			
age	**481.847**	**1**	**<0.001**
dry mass	**7.643**	**1**	**0.006**
treatment	2.055	4	0.726
age × dry mass	2.014	1	0.156
age × treatment	3.276	4	0.513
dry mass × treatment	**13.095**	**4**	**0.011**
age × dry mass × treatment	3.744	4	0.442
Total bufadienolide quantity (TBQ)			
age	**10.341**	**1**	**0.001**
dry mass	2.139	1	0.144
treatment	1.013	4	0.908
age × dry mass	1.233	1	0.267
age × treatment	0.778	4	0.941
dry mass × treatment	1.073	4	0.899
age × dry mass × treatment	1.571	4	0.814
Mass-corrected total bufadienolide quantity (mcTBQ)			
age	**62.605**	**1**	**<0.001**
treatment	1.743	4	0.783
age × treatment	1.206	4	0.877

Table S2: Effects of age, dry mass, predator treatment and their interactions on number of bufadienolide compounds (NBC) of common toad tadpoles (N = 118). We present the parameter estimates of the final LMM model; the 'intercept' refers to control (predator-naïve) tadpoles and the first age class (developmental stage 29). Significant parameters are highlighted in bold.

	Estimate	SE	df	*t*	*p*
intercept	**12.765**	**0.788**	**52**	**16.204**	**<0.0001**
age (dev. stage 42)	**-5.023**	**0.228**	**52**	**-22.017**	**<0.0001**
dry mass (mg)	0.145	0.052	52	2.755	0.008
dragonfly	1.099	0.968	44	1.135	0.262
backswimmer	1.181	0.94	44	1.256	0.216
stickleback	**1.934**	**0.926**	**44**	**2.09**	**0.043**
newt	-0.057	1.081	44	-0.053	0.958
dry mass × dragonfly	-0.071	0.061	52	-1.164	0.25
dry mass × backswimmer	-0.093	0.059	52	-1.579	0.121
dry mass × stickleback	**-0.139**	**0.056**	**52**	**-2.469**	**0.017**
dry mass × newt	0.001	0.069	52	0.012	0.99

Table S3: Effects of the number of common frog tadpoles (*R. temporaria*) eaten, predator size and treatment on survival of toad tadpoles in the predation trials. We present the parameter estimates of the full GEE models; the 'intercept' shows the logit of survival for the control tadpoles and the 'treatment' parameter shows the difference in logit survival between the tadpoles raised with the respective predator and the control tadpoles. Significant terms are highlighted in bold, a marginally non-significant term is marked with an asterisk.

	N	Estimate	SE	Wald χ^2	*P*
dragonflies	23				
intercept		-0.501	0.642	0.61	0.44
frog tadpoles eaten		-0.069	0.141	0.24	0.62
predator size		-0.083	0.067	1.53	0.22
treatment		**1.125**	**0.256**	**19.33**	**<0.001**
backswimmers	22				
intercept		-5.3	3.829	1.916	0.166
frog tadpoles eaten		0.363	0.397	0.835	0.361
predator size*		0.335	0.191	3.065	0.08
treatment		0.725	0.629	1.331	0.249
sticklebacks	23				
intercept		6.505	5.036	1.67	0.2
frog tadpoles eaten		0.195	0.267	0.54	0.46
predator size		-0.119	0.115	1.07	0.3
treatment		0.3	0.796	0.14	0.71

Figures

Fig. S1: Alpha and beta diversity of bufadienolide compounds in toad tadpoles (developmental stage 29) and metamorphs (stage 42). Zero-order (q=0), first-order (q=1) and second-order (q=2) diversity indices, respectively, correspond to the number of toxin compounds (α) or toxin cocktails (β), Shannon entropy, and Simpson's probability of identity. Abbreviations of the treatments are as follows: C: control, D: dragonfly, B: backswimmer, S: stickleback, N: newt.

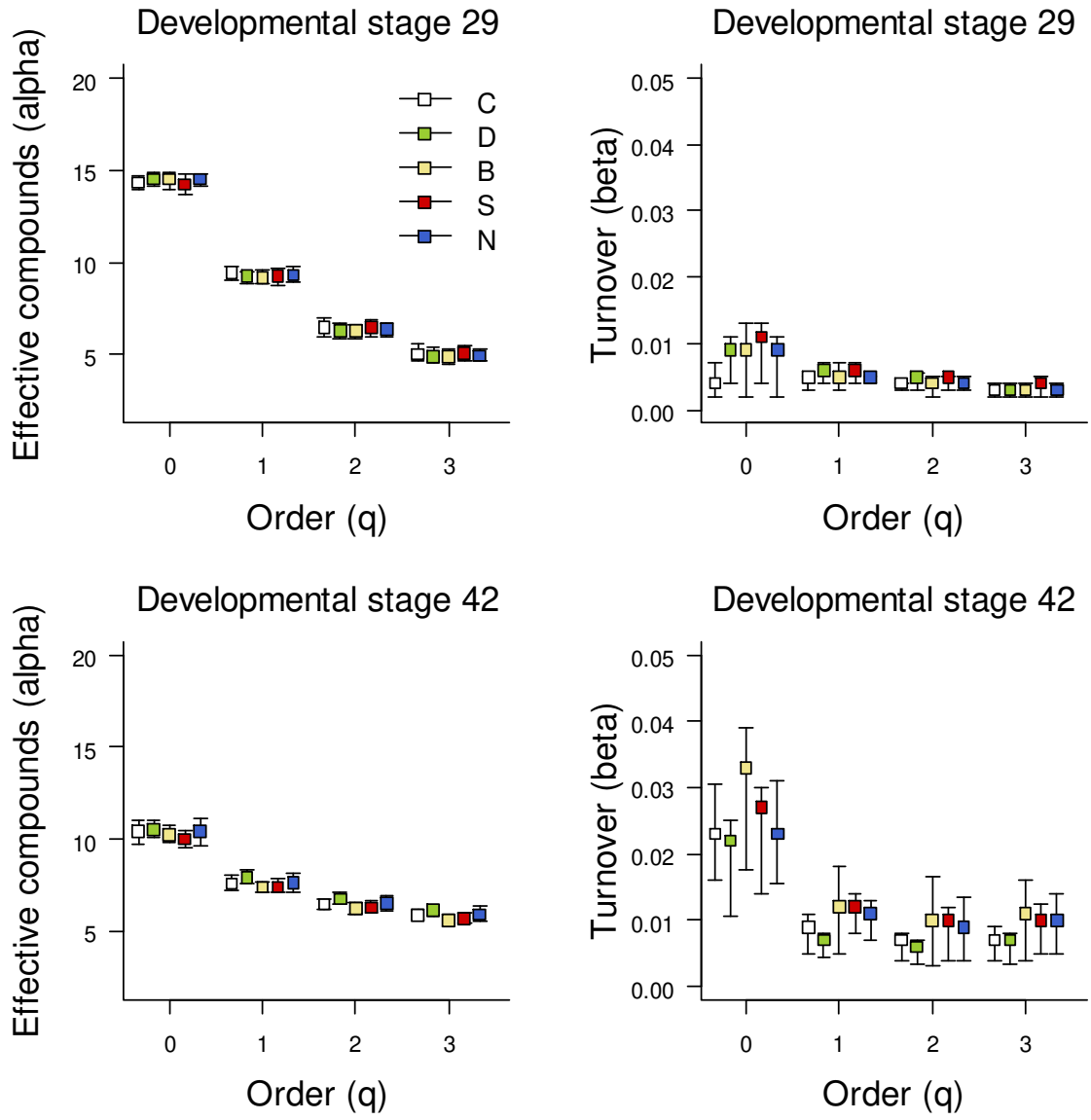


Fig. S2: Number of bufadienolide compounds (NBC) in relation to dry mass of toad tadpoles when reared without predators or in the presence of caged sticklebacks. Regression lines were fitted from the final model presented in Table S2.

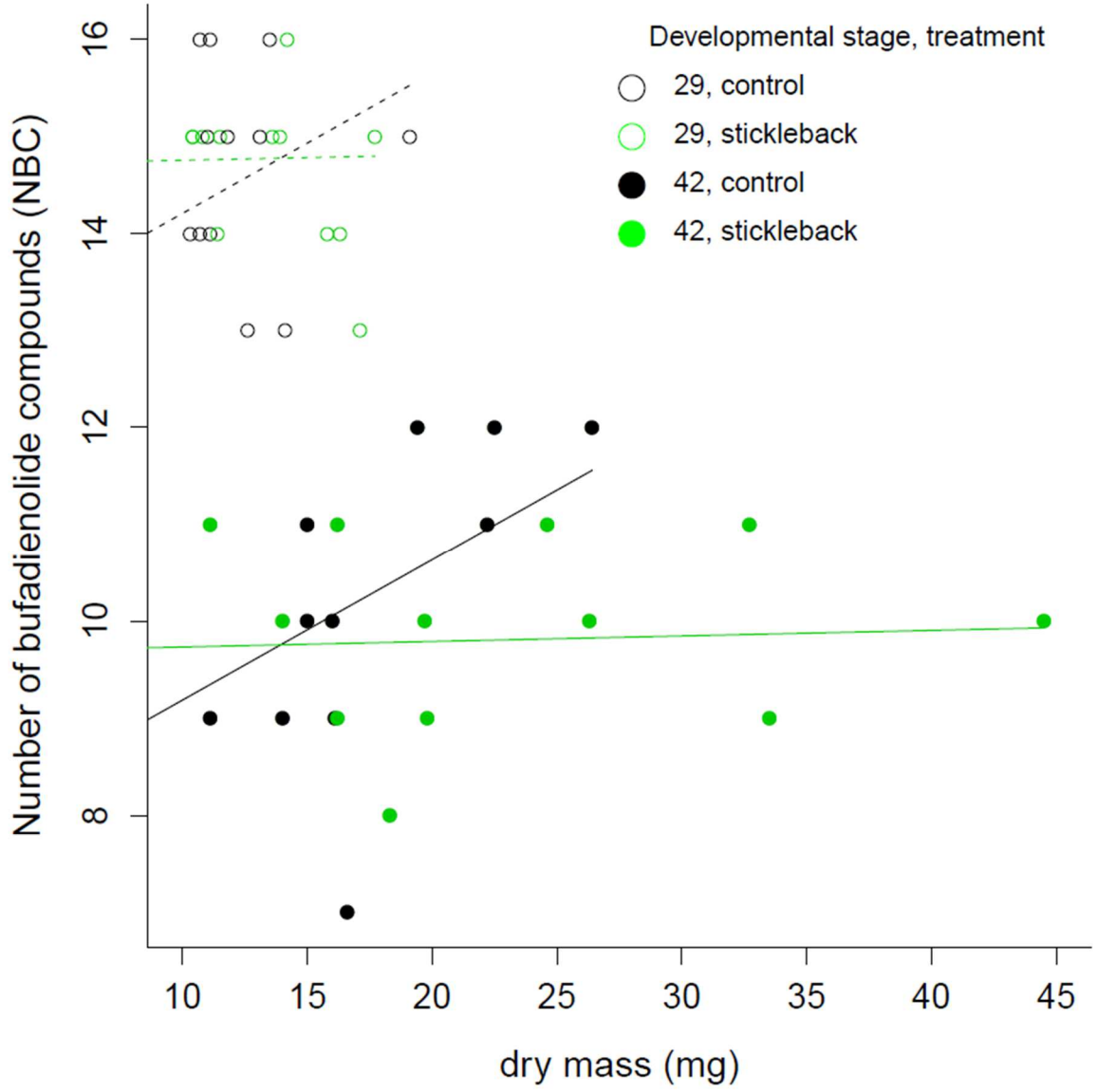


Fig. S3: Effect of predator treatment on length of larval development (mesocosm median number of days until metamorphosis) in common toad tadpoles. A significant difference between control tadpoles and tadpoles reared with fish is marked with asterisks ($P < 0.01$).

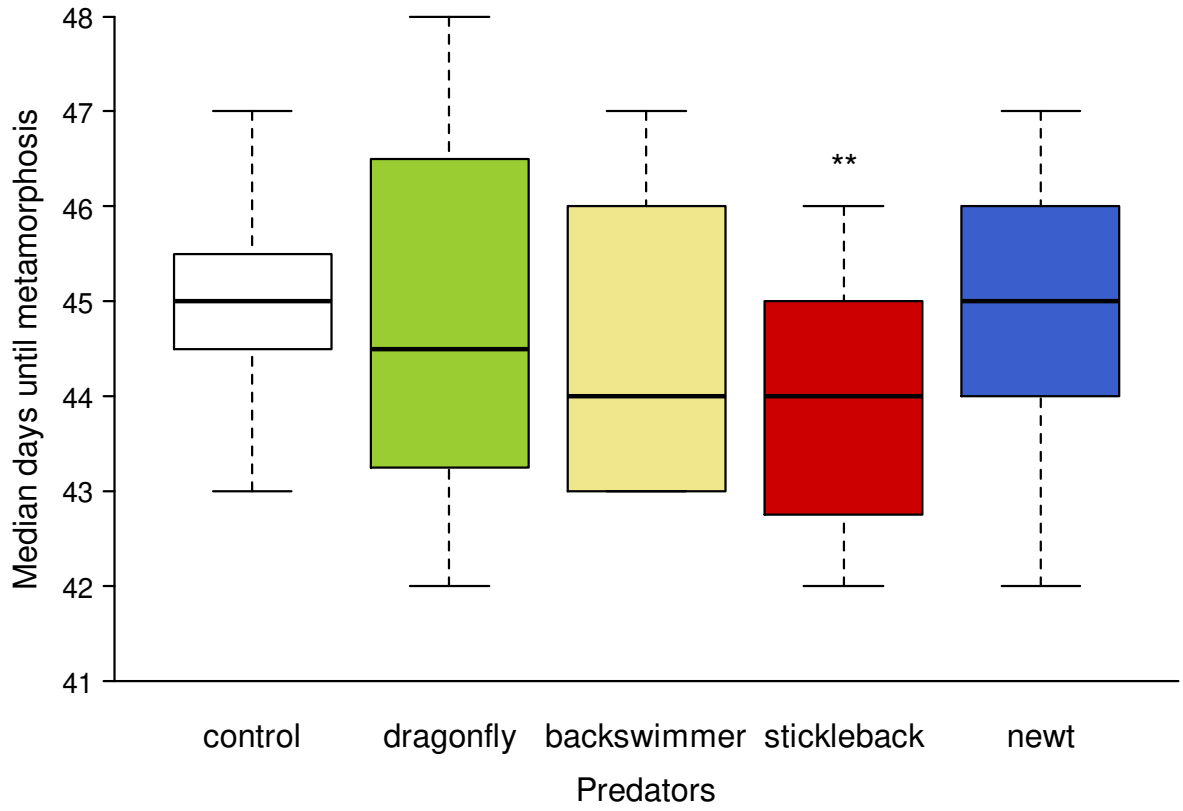
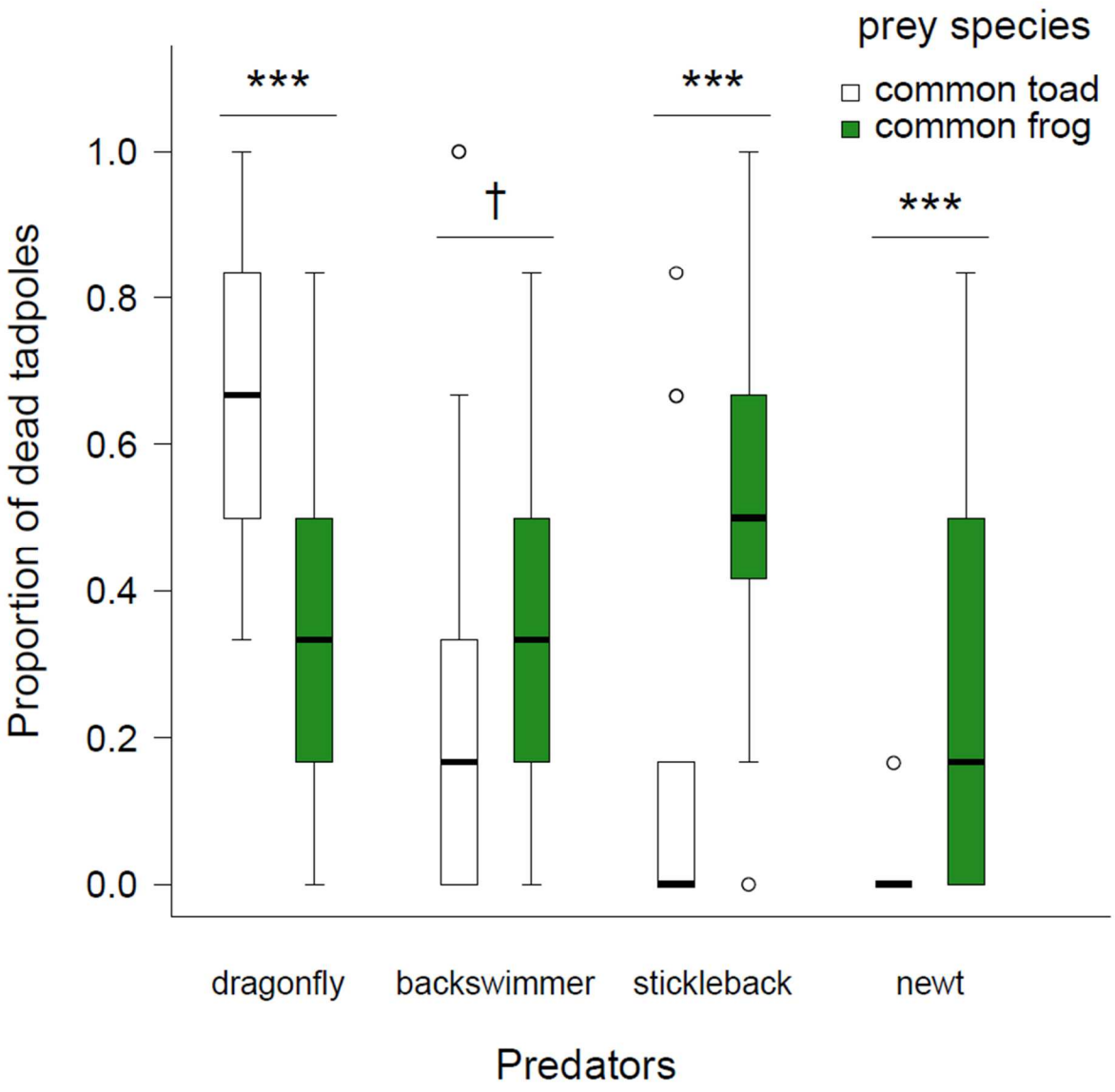


Fig. S4: Proportion of dead tadpoles (palatability) of common toads and common frogs in the predation trials. Palatability was analysed using t-tests in R. Significant differences between the two species are marked with asterisks (p = <0.001), a marginally non-significant difference is marked with a dagger symbol (p = 0.071).



Paper IV

Summary

Inducible defences are ubiquitous in the animal kingdom, but we still know very little about plastic changes in chemical defences in response to predators and the factors favouring their evolution. We tested for predator-induced changes in toxin production of larval common toads (*Bufo bufo*), which are known to synthesize bufadienolide compounds themselves. We also assessed if baseline toxin production and inducibility of chemical defences may vary among populations, and if the intensity of induced responses depended on predator species present by testing individuals originating from three permanent and three temporary ponds and raising larvae in the presence or absence of chemical cues of three predators.

We found that tadpoles raised with chemical cues of predation risk produced higher numbers of bufadienolide compounds and larger total bufadienolide quantities than their predator-naïve conspecifics. Further, the intensity of responses in chemical defence depended on the predator species present. Baseline toxin content and the magnitude of induced responses in toxin production did not differ between tadpoles originating from temporary vs. permanent ponds. Also, the intensity of antipredator responses in total bufadienolide quantity did not vary significantly among the sampled populations. However, we detected significant among-population differences in the magnitude of predator-induced changes in the number of bufadienolide compounds.

These results provide the first compelling evidence for predator-induced changes in chemical defence of a vertebrate that may have evolved to enhance survival probability of responding individuals.

Predator induced chemical defence in a vertebrate: toad tadpoles boost their toxin production in response to carnivores

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Keywords: antipredator defence, anuran amphibian, phenotypic plasticity, poison, variability, genetic assimilation

Introduction

Inducible responses to predators are ubiquitous in the animal kingdom (Tollrian & Harvell 1999). They shape ecological patterns and processes and thereby contribute to the diversity, stability and persistence of communities, populations and species (Miner et al. 2005), and may pave the way for speciation (West-Eberhard 1989, 2003; Pfennig et al. 2010). Such inducible defences evolve because they can enhance fitness when facing natural enemies (Harvell 1990), and can manifest in many forms, including altered behaviour, morphology and life history (Tollrian & Harvell 1999). However, whether animals are capable of plastically adjusting their chemical defences to the actual risk of predation, like many plants do (Karban & Baldwin 1997), has remained little known (Hettyey et al. 2014).

Chemical defences can be found in many animal taxa and toxins can be effective in deterring predators (Toledo and Jared 1995, Kicklighter 2012). Toxicity is known to vary within species among populations and life stages (Kubanek et al. 2002; Fordyce et al. 2006; Hayes et al. 2009; Bókony et al. 2016; Ujszegi et al. 2017; Üveges et al. 2017), which indicates that the physiological machinery of toxin synthesis is flexible and can adapt to predictable spatial and temporal differences in relevant environmental factors. Also, a handful of studies suggest that plastic responses in chemical defences may be induced by the appearance of pathogens (Miele et al. 1998; Mangoni et al. 2001), competitors (Bókony et al. 2016, 2018), and even by anthropogenic pollutants (Bókony et al. 2017). Although similar changes in toxin production in response to predators are known to exist in some lower invertebrates (e.g. a porifer: Ebel et al. 1997; and cnidarians: Slattery et al. 2001; Thornton & Kerr 2002), evidence for vertebrates is scarce and controversial.

Benard and Fordyce (2003) as well as Hagman and colleagues (2009) showed that metamorphic toads (*Anaxyrus boreas* and *Rhinella marina*, respectively), that had been raised in the presence of chemical cues on predation risk during their larval life, altered their bufadienolide toxin synthesis compared to their predator-naïve siblings, but a similar change could not be found in the larvae. However, because predation pressure in the terrestrial habitat of metamorphs is unrelated to that experienced during the aquatic larval stage, the adaptive significance of these environment-induced changes in toxin production is unclear. Further, Üveges et al. (2017) also tested for antipredator responses in toxin synthesis in common toad tadpoles (*Bufo bufo*), but found no effect of chemical cues of predation threat. Predation pressure acting on larval anurans can largely differ between water bodies and may vary between years. Local adaptation can consequently lead to considerable among-population variation in the expression of defences and in the magnitude of its inducible component (Kishida et al. 2007;

Van Buskirk 2014; Hettyey et al. 2016). Because the few studies that exist used individuals originating from only one or two populations, they may have failed in detecting inducible changes in chemical defences due to accidental choice of populations with low levels of inducibility (see Fig. 3 in Hagman et al. 2009). Recently, Bucciarelli and colleagues (2017) reported an increase in the quantity of tetrodotoxin (a highly potent neurotoxin) in *Taricha torosa* newts resulting from repeated invasive skin sampling. Although they claimed that these changes represented predator-induced responses in toxin production, this interpretation remains ambiguous, because no natural predators were used in the experiment, and other environmental stressors can also stimulate the production of chemical defences (Mangoni et al. 2001; Bókony et al. 2017,2018). It also remains to be demonstrated unambiguously whether newts, or indeed metazoans in general, are capable of synthesizing tetrodotoxin or if it is always sequestered from an exogenous source (Chau et al. 2011; Bane et al. 2014; Magarlamov et al. 2017).

To perform a comprehensive test of predator-induced changes in the chemical defences of a vertebrate, we conducted an experiment with an anuran amphibian, the common toad (*Bufo bufo* Linnaeus, 1758), which produces bufadienolide toxins already in early larval stages (Üveges et al. 2017). We collected freshly laid eggs from three permanent and three temporary ponds, reared hatching larvae either in the absence or presence of cues on predation threat and assessed their bufadienolide toxin content after 20 days. We simulated predation threat by exposing developing tadpoles to chemical cues originating from injured conspecifics combined with the chemical cues of either dragonfly larvae, newts or fish. Dragonfly larvae and newts are typical top predators of smaller, temporary water bodies, while fishes dominate permanent ponds and lakes.

We predicted to observe elevated bufadienolide content in tadpoles reared in the presence of cues on predation threat as compared to their predator-naïve conspecifics. We also predicted that variation in the magnitude of induced changes in toxin production would depend on the predator present in the environment of developing tadpoles. Fishes are considered the most voracious predators of anuran larvae in general, followed by Aeshnid dragonfly larvae and newts (Semlitsch 1993; Relyea 2001), and chemical defences of common toad tadpoles appear to be more effective against vertebrate than invertebrate predators (Henrikson 1990; Manteifel & Reshetnikov 2002; Gunzburger & Travis 2005; Üveges et al. unpublished). Further, we expected to find signs of local adaptation to differences in predation risk (Kawecki and Ebert 2004) in the form of habitat dependence of baseline toxin content (i.e. the number and amount of bufadienolides produced when developing in a predator-free environment) and in the intensity of antipredator responses in toxin synthesis. Continuously high predation risk

by fishes in permanent ponds may select for higher baseline toxin production and/or more intense plastic responses than weaker risk in temporary water bodies (for analogous results regarding behavioural and morphological defences see e.g., Magurran 1990; Åbjörnsson et al. 2004; Kishida et al. 2007; Herczeg et al. 2010; Hettyey et al. 2016). At the same time, however, constantly high predation risk may also purge plasticity in toxin production (through genetic assimilation; West-Eberhard, 2003; Crispo, 2007; Pfennig et al. 2010) and high baseline levels of toxin production may hinder a further increase in bufadienolide synthesis because of physiological constraints. Therefore no clear prediction could be formulated regarding the differences in the magnitude or direction of the inducibility of toad chemical defences between permanent and temporary waterbodies.

Methods

Experimental procedures

In early spring 2016 we collected 50 eggs from each of ten common toad egg strings from each of six water bodies located in the Pilis-Visegrádi Mountains, Hungary. Three of these water bodies are permanent ponds inhabited by fish: Apátkúti tó (P1; 47°46'1.55"N, 18°58'53.11"E), Garancsi tó (P2; 47°37'25.38"N, 18°48'26.18"E), and Határréti tó (P3; 47°38'46.90"N, 18°54'31.82"E), while the other three are temporary ponds lacking fish: Jávor tó (T1; 47°42'50.32"N, 19°1'10.74"E), Békás tó (T2; 47°34'34.72"N, 18°52'8.06"E) and Szárazfarkas-belső (T3; 47°44'4.12"N, 18°49'7.04"E). We transferred eggs to the experimental station of the Plant Protection Institute (Centre for Agricultural Research, Hungarian Academy of Sciences) in Budapest, were we kept them in the laboratory separated by families in 0.5 l reconstituted soft water (RSW; 48 mg/l $NaHCO_3$, 30 mg/l $CaSO_4 \times 2\ H_2O$, 61 mg/l $MgSO_4 \times 7H_2O$ and 2 mg/l KCl dissolved in reverse-osmosis filtered tap water and treated with UV) until hatching. We set room temperature to 20°C during daylight hours, which we allowed to decrease at night to 17 °C. Lighting was set to a 13.5:10.5 h light:dark cycle in the beginning, which we modified weekly by lengthening the day by half an hour to closely simulate natural changes in the photoperiod.

Two days after hatching, we haphazardly selected 4 healthy-looking tadpoles of each family, placed them individually into 2 l rearing containers filled with 0.7 l RSW, and assigned them randomly to treatments. Containers were arranged in a full factorial design with stratified randomisation, where each block contained one tadpole from each pond and predator treatment combination. In each block families were represented only once. We exposed one member per family to each of the four treatments (one control and three predator treatments, see below),

resulting in a total of 240 experimental units. We changed water twice a week and fed tadpoles on these occasions with a 1:100 mixture of finely ground Spirulina powder and slightly boiled spinach *ad libitum*.

As predators we used six 4th instar larvae of the emperor dragonfly (*Anax imperator*), six adult males of the smooth newt (*Lissotriton vulgaris*) and six adults of the European perch (*Perca fluviatilis*). We housed predators under the same temperature and lighting conditions as the experimental tadpoles, and we changed their water twice a week. To ensure similar concentrations of chemical cues in the three predator treatments, we adjusted the quantity of water and food provided to predators as detailed below. We kept dragonfly larvae individually in 2 l containers filled with 1 l RSW and equipped with a plastic perching stick. Perches were housed together in a 140 l tub filled with 105 l aerated RSW (which was later lowered to 95 l, see below). We maintained newts together in a 40 l container filled with 8 l RSW. This way, the body mass of predators for every litre of RSW was the same across predator species (mean ± SD: 1.344 ± 0.021 g/l at the beginning of the experiment). When replacing some predators during the experiment (because they refused to eat (dragonfly larvae), moulted to the terrestrial form (newts), or spawned (perch)), we took care to use similar-sized individuals. In order to maintain balanced body mass by water volume ratios (mean ± SD: 1.351 ± 0.041 g/l at termination of the experiment), we re-adjusted the water level of fishes halfway through the experiment. We fed predators with one agile frog (*Rana dalmatina*) tadpole (a preferred prey of all three predator species) for every 2 l of RSW five times a week and with ca. 10 sludge worms (*Tubifex sp.*) for every 2 liters of RSW as supplementary food twice a week. Thus, dragonfly larvae received three tadpoles (alternating between individuals at each feeding session), newts four tadpoles, and perch 52 tadpoles (47 after re-adjustment of the water level). We did not weigh tadpoles used as food for predators, but chose similar sized individuals at each feeding. We fed predators with agile frog tadpoles, because we wanted to ensure that focal tadpoles in different predator treatments received equal concentrations of prey-borne cues, while the predators differ in how readily they feed on toad tadpoles: smooth newts are reluctant to feed on common toad tadpoles, European perch can live on toad larvae, but avoid them if possible, while emperor dragonfly larvae readily feed solely on toad larvae (Henrikson 1990; Manteifel & Reshetnikov 2002; Üvegeset al. unpublished). We provided chemical cues originating from injured and killed conspecifics by adding homogenized toad tadpoles to the stimulus water, as follows.

We prepared stimulus water containing both predator-borne and prey-borne chemical cues on predation risk (to induce intense antipredator responses, see e.g., Hettyey et al. 2015)

for each predator species separately, by homogenizing 138.509 ± 2.444 mg (mean ± SD) common toad tadpoles using a blender in approx.150 ml water. We did not anesthetize the tadpoles prior to homogenization, because we did not want tricaine-methanesulfonate, a general anaesthetic of tadpoles, to interfere with the chemical analysis. However, because the homogenisation procedure is very fast, we believe that tadpoles suffered only for a brief amount of time, much shorter than what they may experience in nature (e.g. following a dragonfly attack, pers. obs.). Afterwards, we added the homogenate to 2 l water taken from the housing container(s) of each type of predator. Five times a week we pipetted 20 ml freshly prepared stimulus water into rearing containers of focal tadpoles assigned to the respective predator treatments. Simultaneously, we added 20 ml RSW into rearing containers of control tadpoles. Because of almost instant death, tadpoles homogenized using a blender may not produce or release all types of chemical cues at the same quantity as during a natural predation event (Fraker et al. 2009), but similar methods have been successfully used before and resulted in strong induced responses in focal tadpoles (Schoeppner and Relyea 2005; Hagman et al 2009; Hettyey et al. 2015). The procedure described above resulted in 1.657 ± 0.029 mg conspecific tadpole tissue L^{-1} (mean ± SD) in the rearing containers of focal tadpoles. Similar and also lower concentrations of chemical cues of predation threat (Van Buskirk & Arioli 2002; McCoy et al. 2012; Hettyey et al. 2015) have been shown to induce plastic responses in amphibian larvae. After preparation of stimulus water, we filled containers of predators with RSW to the original level.

To be able to later assess treatment effects on toxin content of focal toad tadpoles, we conserved all 240 individuals in HPLC-grade absolute methanol 20 days after start of the experiment, when tadpoles were at developmental stage 35 (Gosner 1960). We chose a duration of approximately three weeks to allow tadpoles enough time for mounting a response in toxin production, for growing to a relatively large size facilitating the quantification of toxin content, and because bufadienolide content of common toad tadpoles is highest in well-developed, ca. 3 weeks old larvae (Üveges et al. 2017; Ujszegi et al. 2017). No tadpole died before termination of the experiment.

Analysis of toxin content

Preparation of samples and analysis of bufadienolide content of toads was carried out using high-performance liquid chromatography with diode-array detection and mass spectrometry (HPLC-DAD-MS) according to already published protocols (Bókony *et al.*, 2016; Bókony *et al.*, 2017; Üveges *et al.*, 2017; Bókony *et al.*, 2018), with the exception that we also recorded

full scan spectra in the range of 350–800 *m/z* and also performed selected-ion monitoring (SIM) acquisition detecting the base peak of bufadienolides we previously found in common toads.

Statistical analysis

We lost one sample during preparation for HPLC-DAD-MS analysis, resulting in a sample size of 239 tadpoles. We calculated the number of bufadienolide compounds (NBC) for each sample assuming a compound to be present if its signal to noise ratio was at least 3 in the chromatogram. We estimated the quantity of each compound from the area of chromatogram peaks using the calibration curve of the bufotalin standard and obtained estimates of total bufadienolide quantity (TBQ) for each sample by summing up these values (for details see Üveges et al. 2017). This approach only yields rough estimates of TBQ, but due to the unavailability of most bufadienolid standards there is currently no better alternative for toxin quantification, and this method has been successfully applied in similar studies (Benard & Fordyce 2003; Hagman et al. 2009; Bókony et al. 2016; Üveges et al. 2017).

To assess among-population differences in baseline toxin production, we compared the toxin content of tadpoles reared in the control treatment. We tested for potential among-population variation in baseline NBC and TBQ using linear mixed-effects models (LMM) with population as a fixed factor and block as a random factor. Family was not included as a random factor in this analysis, because each family was represented by only one individual in the control treatment. Subsequently, we compared NBC and TBQ between permanent and temporary ponds by calculating a linear contrast from each model.

To test for plasticity in toxin production we analysed variation in NBC and TBQ using LMMs, entering predator treatment as a fixed factor and block, crossed with family nested into population ((block) + (population/family)) as random factors. We assessed among-treatment differences in toxin production between the control and each predator treatment within each population using linear contrasts calculated from full models. In a second step we calculated the difference between permanent and temporary ponds in the response to each predator (i.e. difference between the control and the respective predator treatment), as a linear contrast of the within-population contrasts. Separately, we also used linear contrasts to calculate estimates of among treatment differences irrespective of population of origin. Analyses of TBQ corrected for body mass delivered qualitatively similar results (for details see Table S1 & S2).

We confirmed that our data fit the assumptions of analyses by inspecting residual plots. We ran statistical analyses in R v. 3.4.0 (R Development Core Team 2017) using the 'lmer' function of the 'lme4' package (Bates et al. 2015). We obtained *P*-values for LMMs from

anova-tables with type-3 sums-of-squares using the 'anova' function of the 'lmerTest' package (Kuznetsova et al. 2017). Satterthwaite approximation was used to calculate degrees of freedom. For calculating linear contrasts we used the 'lsmeans' package (Lenth 2016) and adjusted *P*-values applying the false discovery rate method (Benjamini & Hochberg 1995). We report least-squares means with standard errors (SE).

Results

Baseline toxin content

The analysis on control tadpoles reared in the absence of cues of predation threat did not reveal significant variation among populations either in NBC (LMM; $F_{5,45} = 1$, $P = 0.43$; Fig. 1A) or in TBQ ($F_{5,47.3} = 0.73$, $P = 0.6$; Fig. 1B). As indicated by linear contrasts, baseline NBC and TBQ also did not differ between tadpoles originating from permanent and temporary ponds (difference in NBC: 0.37 ± 0.26, $t_{45} = 1.44$, $P = 0.16$; in TBQ: 293.06 ± 331.67 ng / tadpole, $t_{66.03} = 0.88$, $P = 0.38$).

Plasticity in toxin production

Tadpoles exposed to different predator treatments responded with the production of increased numbers of bufadienolide compounds (LMM; $F_{3,171.8} = 35.78$, $P < 0.001$; Fig. 2; Fig S1A). Predator-naïve tadpoles produced the lowest NBC (18.15 ± 0.11 compounds), while the presence of chemical cues on predation resulted in higher NBC (in the presence of dragonflies: 19.3 ± 0.11; newts: 19.05 ± 0.11; perch: 19.55 ± 0.11, Fig. S1A). Linear contrasts revealed that predator-naïve tadpoles contained significantly lower NBC as compared to tadpoles exposed to cues of any type of predator (control vs. newt: $t_{172,1} = 6.29$, $P < 0.001$; control vs. dragonfly: $t_{171,5} = 7.86$, $P < 0.001$; control vs. perch: $t_{171,5} = 9.76$, $P < 0.001$; Table S3, Fig. S1A). We detected significant variation among tadpoles according to population of origin in the intensity of predator-induced changes in NBC (based on non-overlapping confidence intervals, see Fig. 2). When analysing antipredator responses in NBC in tadpoles originating from the three temporary and the three permanent ponds together, we found significant changes in response to all predators in both types of water bodies, but these responses did not differ between tadpole populations originating from the two pond types (temporary vs. permanent ponds; see Table 1).

Total bufadienolide quantity also varied among predator treatments (LMM; $F_{3,152.2} = 10.96$, $P < 0.001$; Fig. 2; Fig. S1B). Tadpoles in the control treatment produced the lowest TBQ (4155.7 ± 199 ng / tadpole; mean ± SE), those reared in the presence of cues from perch the highest (5240.1 ± 199), whereas tadpoles exposed to cues of dragonflies and newts contained

intermediate toxin levels (dragonflies: 4697.6 ± 199; newts: 4655.4 ± 200; Fig. S1B). Linear contrasts indicated that predator-naïve tadpoles had lower TBQ than tadpoles in any other treatment (control vs. newt: $t_{189,1} = 2.63$, $P = 0.011$; control vs. dragonfly: $t_{188,7} = 2.86$, $P = 0.007$; control vs. perch: $t_{188,7} = 5.73$, $P < 0.001$; Table S3, Fig. S1B). We did not detect variation in TBQ according to population of origin (Fig. 2). When analysing antipredator responses in TBQ in tadpoles originating from the three temporary and the three permanent ponds together, we did not find significant changes in response to newts in either type of water body and in response to dragonflies in permanent ponds, while tadpoles originating from temporary ponds responded to dragonflies with increased toxin production, and so did tadpoles originating from both types of water bodies exposed to chemical cues of perch (Table 1, Fig. 2). However, linear contrasts did not reveal significant differences in the magnitude of responses between tadpoles originating from temporary and permanent ponds (Table 1).

Discussion

We document predator-induced changes in the chemical defence of common toad larvae. Tadpoles reared in the presence of chemical cues on predation threat produced a larger number of bufadienolide compounds and higher total bufadienolide quantity as compared to tadpoles that developed in a predator-free environment. Furthermore, the strength of induced responses depended on the type of predator present in the larval environment (see Table S3 and Fig. S1). We did not find evidence for local adaptation in toxin production in baseline toxin content, nor did groups of tadpoles vary in their antipredator response in total bufadienolide quantity according to their pond of origin. However, the magnitude of predator-induced responses in the number of bufadienolide compounds varied significantly among populations. Neither baseline toxin content, nor the magnitude of induced responses in chemical defence differed significantly between tadpoles originating from permanent and temporary ponds.

Our study is the first to deliver clear evidence for predator-induced changes in the chemical defence of a vertebrate which can be interpreted as adaptive phenotypic plasticity. Although it has been known for more than a decade that invertebrates can plastically adjust their toxin production to the presence of predators in ways that can enhance their survival probabilities (e.g., Ebel et al 1997; Slattery et al. 2001; Thornton & Kerr 2002), similar reports for vertebrates have so far provided only circumstantial evidence (Benard & Fordyce 2003; Hagman et al. 2009; Bucciarelli et al. 2017). Nonetheless, further experimental investigation is required to test if enhanced toxin production indeed provides additional protection against

predators and, hence, the observed induced changes in toxin production represent a case for adaptive plasticity in chemical defence.

Our results also provide support for the hypothesis that the intensity of induced changes in chemical defences can vary depending on the predator species present, very much like in other defensive traits (Sih 1986; Relyea 2001; Van Buskirk and Arioli 2002; Hettyey et al. 2011). Toad tadpoles produced the highest number and quantity of bufadienolide compounds in the presence of perch, while they responded weaker to dragonfly larvae and least to newts. Fishes are in general the most voracious predators of anuran larvae, but vertebrate predators appear to be more sensitive to the toxins produced by toad tadpoles than invertebrate predators, such as dragonfly larvae (Henrikson 1990; Manteifel & Reshetnikov 2002; Gunzburger & Travis 2005; Üveges et al. unpublished). Nonetheless, besides the danger posed by a predator, the effectiveness of the induced defence against that specific predator is likely to be equally important for the adjustment of the response (Sih et al. 2011). Intensifying toxin synthesis against an enemy that is immune to its toxic effects would not benefit the prey and can even be maladaptive due to costs of enhanced toxin production or storage. However, the production of bufadienolides may not be costly in terms of energy expenditure (Kurali et al. 2016, Üveges et al.2017, Bókony et al. 2018). Our study using three different predators was designed to maximize the chances of detecting inducible antipredator responses in chemical defences. We provided both predator- and prey-borne chemical cues in equal concentrations across predator treatments to focal individuals. On the other hand, we did not experimentally investigate tadpole survival in the presence of free-ranging predators. Consequently, we cannot conclude on the interrelationship between predator dangerousness, the effectiveness of chemical defences and the magnitude of induced responses in toxin production. Uncovering these relationships will be important for understanding the evolutionary origin and maintenance of inducible responses in chemical defences.

Our result that predation threat can induce changes in the toxin production of common toad tadpoles contradicts the finding of a previous study (Üveges et al. 2017). How can this discrepancy be explained? Populations can vary in how plastically they respond to environmental cues (Magurran 1990; Åbjörnsson et al. 2004; West-Eberhard, 2003; Crispo, 2007; Pfennig et al. 2010; Hettyey et al. 2016) and in the present study we provide evidence that this is also true for the strength of antipredator responses in toxin production. In the previous experiment we may have accidentally used a population exhibiting low levels of plasticity in chemical defences. Also, large differences among studies in sample sizes (60 samples per treatment in the present study vs. 10 replicates per treatment in the previous

investigation) may have resulted in differences in statistical power, contributing to our inability of detecting treatment effects in our previous study and enhancing our capability to detect them in the present experiment. Finally, in the aforementioned study we raised tadpoles in relatively dense groups (3 tadpoles in 1.5 l), whereas in the present study we reared tadpoles individually. It is known that the presence of conspecifics in the environment can affect the expression of inducible defences due to prey risk assessment taking into account risk dilution and group vigilance (Peacor 2003; Van Buskirk et al. 2011; Tollrian et al. 2015). Further, we recently showed that common toad tadpoles adjust their toxin production to the density of conspecifics even in the absence of predators (Bókony et al. 2018). Nonetheless, it remains to be tested experimentally if differences in the social environment influence plastic anti-predator responses in chemical defence.

We found mixed evidence for local adaptation in chemical defences of toad tadpole populations: the magnitude of induced antipredator responses in the number of bufadienolide compounds varied significantly among the six sampled populations, while similar variation was not detectable in baseline toxin content and in the extent of induced changes in total bufadienolide quantity. The lack of systematic differences between groups of populations originating from temporary or permanent ponds was somewhat surprising, because fishes are in general considered the most voracious predators of anuran larvae (Semlitsch 1993; Relyea 2001), and failure to produce sufficiently effective defences may lead to very low survival probability in fish-infested permanent ponds. Indeed, populations exposed to continuously high predation risk have been shown to exhibit more defended phenotypes and more intense antipredator responses in behaviour, morphology and life history than populations in low-risk habitats (Magurran 1990; Åbjörnsson et al. 2004; Kishida et al. 2007; Herczeg et al. 2010; Hettyey et al. 2016). The incongruity between former empirical evidence and our results regarding the comparison between populations originating from temporary vs. permanent ponds may be accounted to gene flow between permanent ponds and adjacent temporary puddles obstructing local adaptation to varying levels of predation risk (Kawecki & Ebert 2004; Yeaman & Otto 2011; Blanquart et al. 2012). Also, shallow areas inaccessible to fishes may provide suitable refugia for tadpoles in fish-containing ponds, weakening selection acting towards fixation of high levels of toxin production and of plasticity. Finally, chemical defences of toads are in general more effective against vertebrate than invertebrate predators (Henrikson 1990; Manteifel & Reshetnikov 2002; Gunzburger & Travis 2005), and already relatively low quantities of bufadienolides may provide efficient defences against fishes (Üveges et al. unpublished). Contrary to theory (West-Eberhard, 2003; Crispo, 2007; Pfennig et al. 2010) on

the other hand, we also did not find unequivocal evidence for the fixation of induced defences in permanent ponds, where fishes are permanently present. Consequently, ecological factors other than fish presence, such as the density of other predators or of conspecifics, may be more important in determining the strength of selection on chemical defences and on plasticity therein.

In conclusion, our study provides clear evidence for inducible responses to predators in chemical defences of a vertebrate. Importantly, our findings are likely to reflect the outcome of concurrent natural selection because we observed inducible changes in toxin synthesis manifesting in the same environment in which study organisms experienced cues on predation threat, and also because the observed changes were induced by predators that co-occur with common toad tadpoles in natural populations. Nonetheless, it remains an open question if antipredator responses in toxin synthesis of toad tadpoles are indeed adaptive, and how widespread predator-induced changes in chemical defences occur in the animal kingdom.

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Table 1: Treatment effects on the number of bufadienolide compounds (NBC) and total bufadienolide quantity (TBQ) in tadpole populations originating from temporary (T) and permanent (P) ponds. Estimates of linear contrasts compare tadpoles reared in the control treatment to those exposed to chemical cues of newts, dragonfly larvae or perch, within each population type, i.e. permanent (P) or temporary (T) ponds. *P*-values were corrected for false discovery rate. We also present comparisons of the effects of predator treatment (i.e. the difference between control and predator treatment) between permanent and temporary ponds (P vs. T) based on linear contrasts of the within-population contrasts. Significant differences are highlighted in bold.

Trait	Contrasts	Pond type	Estimate ± SE	df	84% CI	t-ratio	*P*
NBC	Control vs. newt	**T**	**0.98 ± 0.20**	**157.17**	**0.699-1.258**	**4.95**	**<0.001**
		P	**0.80 ± 0.20**	**156.01**	**0.524-1.076**	**4.09**	**<0.001**
		P vs. T	0.18 ± 0.28	156.60	-0.214-0.572	0.64	0.522
	Control vs. dragonfly	**T**	**1.33 ± 0.20**	**156.01**	**1.057-1.610**	**6.81**	**<0.001**
		P	**0.87 ± 0.20**	**156.01**	**0.590-1.143**	**4.43**	**<0.001**
		P vs. T	0.47 ± 0.28	156.01	0.076-0.858	1.69	0.094
	Control vs. perch	**T**	**1.50 ± 0.20**	**156.01**	**1.224-1.776**	**7.66**	**<0.001**
		P	**1.23 ± 0.20**	**156.01**	**0.957-1.510**	**6.30**	**<0.001**
		P vs. T	0.27 ± 0.28	156.01	-0.124-0.658	0.96	0.337
TBQ	Control vs. newt	T	470.4 ± 273.0	123.82	84.4-856.3	1.72	0.087
		P	517.9 ± 269.8	123.29	136.4-899.3	1.92	0.087
		P vs. T	-47.5 ± 383.9	123.56	-590.1-495.1	-0.12	0.902
	Control vs. dragonfly	**T**	**614.8 ± 269.8**	**123.29**	**233.3-996.2**	**2.28**	**0.049**
		P	469.0 ± 269.8	123.29	87.5-850.4	1.74	0.085
		P vs. T	145.8 ± 381.6	123.29	-393.6-685.3	0.38	0.703
	Control vs. perch	**T**	**1265.9 ± 269.8**	**123.29**	**884.5-1647.4**	**4.69**	**<0.001**
		P	**902.8 ± 269.8**	**123.29**	**521.3-1284.3**	**3.35**	**0.001**
		P vs. T	363.1 ± 381.6	123.29	-176.3-902.6	0.95	0.343

Fig. 1: Baseline toxin levels of toad tadpoles. The number of bufadienolide compounds (panel A) and total bufadienolide quantity (panel B) in control tadpoles reared in the absence of cues of predation threat separated by their population of origin. Thick lines represent the median, boxes the interquartile range and whiskers the minimum-maximum range. Numbering of permanent (P) and temporary (T) ponds of origin corresponds with that in the 'Methods' section.

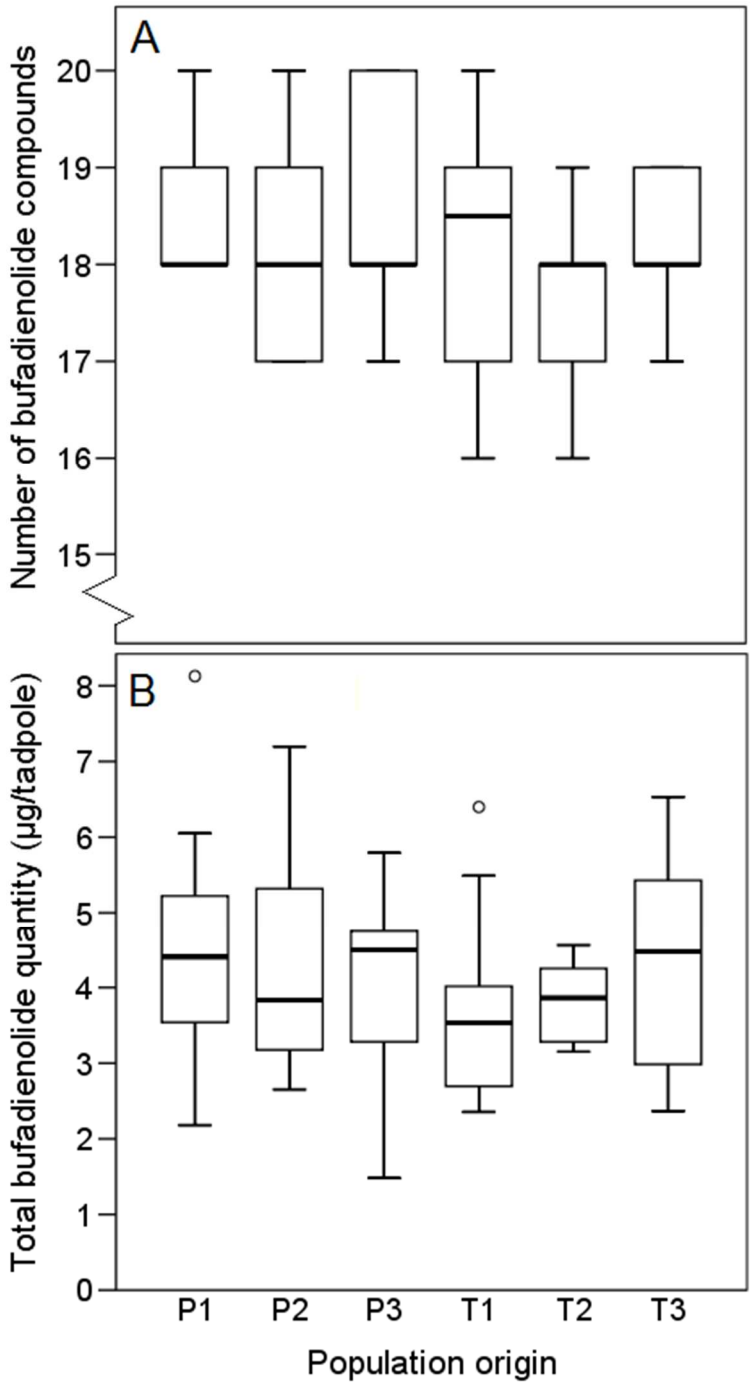
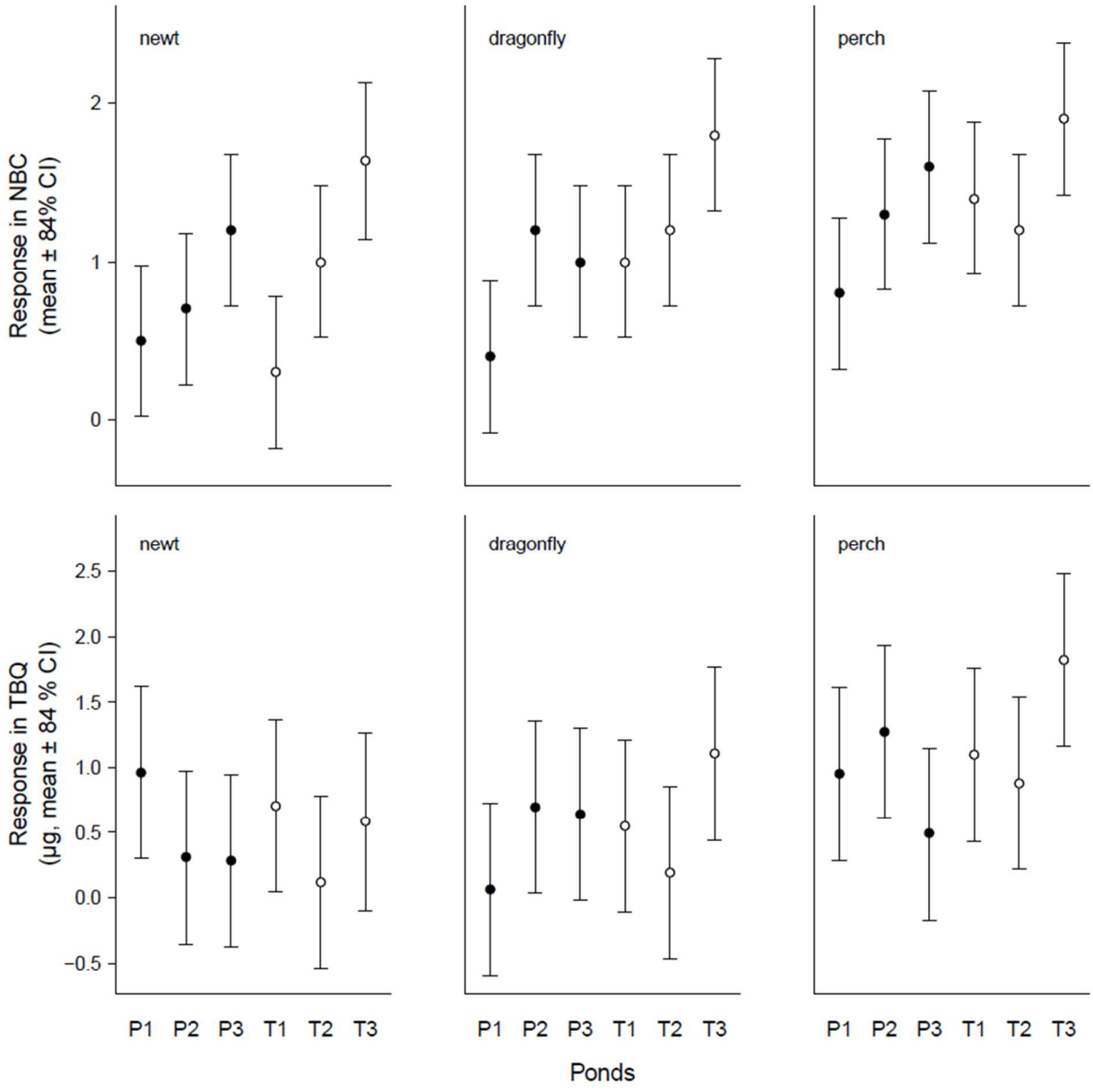


Fig. 2: Predator-induced chemical defence of toad tadpoles. The intensity of antipredator responses in the number of bufadienolide compounds (NBC) and in total bufadienolide quantity (TBQ) in tadpoles originating from three permanent (P) and three temporary (T) ponds. Response intensity was estimated from differences in toxin production between the control and each predator treatment within each population using linear contrasts calculated from LMMs. Means and 84 % confidence intervals (CI) are given. Groups with non-overlapping CI differ from each other significantly.



Supplementary Information for **Paper IV**

Table S1: Estimates of linear contrasts and their *P*-values corrected for false discovery rate, comparing the number of bufadienolide compounds (NBC) and total bufadienolide quantity (TBQ) between tadpoles reared in the control treatment and those exposed to chemical cues of newts, dragonfly larvae or perch, within each population originating from permanent (P) or temporary (T) ponds. In the case of mass-corrected total bufadienolide quantity (mcTBQ) values back-transformed from the logarithmic scale are presented. Significant differences are highlighted in bold.

Trait	Contrasts	Pond of origin	Estimate ± SE	df	84% CI	t-ratio	*P*
NBC	Control vs. newt	P1	0.5 ± 0.34	156.01	0.02-0.98	1.47	0.170
		P2	0.7 ± 0.34	156.01	0.22-1.18	2.06	0.061
		P3	**1.2 ± 0.34**	**156.01**	**0.72-1.68**	**3.54**	**0.002**
		T1	0.3 ± 0.34	156.01	-0.18-0.78	0.88	0.378
		T2	**1.0 ± 0.34**	**156.01**	**0.52-1.48**	**2.95**	**0.007**
		T3	**1.6 ± 0.35**	**159.36**	**1.14-2.13**	**4.68**	**<0.001**
	Control vs. dragonfly	P1	0.4 ± 0.34	156.01	-0.08-0.88	1.18	0.240
		P2	**1.2 ± 0.34**	**156.01**	**0.72-1.68**	**3.54**	**0.001**
		P3	**1.0 ± 0.34**	**156.01**	**0.52-1.48**	**2.95**	**0.004**
		T1	**1.0 ± 0.34**	**156.01**	**0.52-1.48**	**2.95**	**0.004**
		T2	**1.2 ± 0.34**	**156.01**	**0.72-1.68**	**3.54**	**0.001**
		T3	**1.8 ± 0.34**	**156.01**	**1.32-2.28**	**5.31**	**<0.001**
	Control vs. perch	**P1**	**0.8 ± 0.34**	**156.01**	**0.32-1.28**	**2.36**	**0.020**
		P2	**1.3 ± 0.34**	**156.01**	**0.82-1.78**	**3.83**	**<0.001**
		P3	**1.6 ± 0.34**	**156.01**	**1.12-2.08**	**4.72**	**<0.001**
		T1	**1.4 ± 0.34**	**156.01**	**0.92-1.88**	**4.13**	**<0.001**
		T2	**1.2 ± 0.34**	**156.01**	**0.72-1.68**	**3.54**	**<0.001**
		T3	**1.9 ± 0.34**	**156.01**	**1.42-2.38**	**5.60**	**<0.001**
TBQ	Control vs. newt	P1	959.8 ± 467.4	123.29	299.1-1620.5	2.05	0.253
		P2	306.9 ± 467.4	123.29	-353.8-967.6	0.66	0.649
		P3	286.9 ± 467.4	123.29	-373.7-947.6	0.61	0.649
		T1	706.4 ± 467.4	123.29	45.7-1367.1	1.51	0.400
		T2	121.0 ± 467.4	123.29	-539.7-781.7	0.26	0.796
		T3	583.7 ± 483.7	124.83	-100-1267.3	1.21	0.460
	Control vs. dragonfly	P1	63.0 ± 467.4	148.13	-597.7-723.7	0.14	0.893
		P2	699.4 ± 467.4	148.13	38.7-1360.1	1.50	0.341
		P3	644.6 ± 467.4	148.13	-16.1-1305.3	1.38	0.341
		T1	548.9 ± 467.4	148.13	-111.8-1209.5	1.17	0.364
		T2	190.9 ± 467.4	148.13	-469.8-851.6	0.41	0.821
		T3	1104.7 ± 467.4	148.13	444.0-1765.4	2.36	0.118
	Control vs. perch	P1	948.4 ± 467.4	123.29	287.7-1609.1	2.03	0.067
		P2	**1270.6 ± 467.4**	123.29	609.9-1931.3	**2.72**	**0.023**
		P3	489.4 ± 467.4	123.29	-171.3-1150.1	1.05	0.297
		T1	**1094.9 ± 467.4**	123.29	434.2-1755.6	**2.34**	**0.042**
		T2	877.4 ± 467.4	123.29	216.7-1538.1	1.88	0.075
		T3	**1825.4 ± 467.4**	123.29	1164.7-2486.1	**3.91**	**<0.001**
mcTBQ	Control vs. newt	P1	1.190 ± 1.213	150.73	0.906-1.563	0.90	0.618
		P2	1.051 ± 1.213	150.73	0.800-1.380	0.26	0.835
		P3	1.217 ± 1.213	150.73	0.927-1.598	1.02	0.618
		T1	1.172 ± 1.213	150.73	0.892-1.539	0.82	0.618
		T2	1.041 ± 1.213	150.73	0.793-1.367	0.21	0.835
		T3	1.310 ± 1.220	154.12	0.989-1.735	1.36	0.618
	Control vs. dragonfly	**P1**	**1.534 ± 1.213**	**150.73**	**1.168-2.014**	**2.22**	**0.032**

Table S1 continued.

Trait	Contrasts	Pond of origin	Estimate ± SE	df	84% CI	t-ratio	*P*
mcTBQ	Control vs. dragonfly	**P2**	**1.625 ± 1.213**	**150.73**	**1.238-2.134**	**2.52**	**0.026**
		P3	**1.647 ± 1.213**	**150.73**	**1.254-2.162**	**2.56**	**0.026**
		T1	**1.539 ± 1.213**	**150.73**	**1.172-2.020**	**2.23**	**0.032**
		T2	**1.519 ± 1.213**	**150.73**	**1.157-1.995**	**2.17**	**0.032**
		T3	**2.311 ± 1.213**	**150.73**	**1.760-3.034**	**4.34**	**<0.001**
	Control vs. perch	**P1**	**1.628 ± 1.213**	**150.73**	**1.240-2.138**	**2.53**	**0.013**
		P2	**2.457 ± 1.213**	**150.73**	**1.871-3.227**	**4.66**	**<0.001**
		P3	**2.007 ± 1.213**	**150.73**	**1.528-2.635**	**3.61**	**<0.001**
		T1	**2.912 ± 1.213**	**150.73**	**2.217-3.823**	**5.54**	**<0.001**
		T2	**1.863 ± 1.213**	**150.73**	**1.419-2.446**	**3.23**	**0.002**
		T3	**3.576 ± 1.213**	**150.73**	**2.723-4.696**	**6.61**	**<0.001**

Table S2: Treatment effects on mass-corrected total bufadienolide quantity (mcTBQ) in tadpole populations originating from temporary (T) and permanent (P) ponds. Estimates of linear contrasts compare tadpoles reared in the control treatment to those exposed to chemical cues of newts, dragonfly larvae or perch, within each population type, i.e. permanent (P) or temporary (T) ponds. *P*-values were corrected for false discovery rate. We also present comparisons of the effects of predator treatment (i.e. the difference between control and predator treatment) between permanent and temporary ponds (P vs. T) based on linear contrasts of the within-population contrasts. Values are back-transformed from the logarithmic scale.

Trait	Contrasts	Pond type	Estimate ± SE	df	84% CI	t-ratio	*P*
mcTBQ	Control vs. newt	T	1.17 ± 1.12	151.91	0.997-1.371	1.39	0.211
		P	1.15 ± 1.12	150.73	0.983-1.346	1.26	0.211
		P vs. T	1.02 ± 1.17	151.33	0.813-1.271	0.10	0.918
	Control vs. dragonfly	**T**	**1.76 ± 1.12**	**150.73**	**1.499-2.053**	**5.05**	**<0.001**
		P	**1.60 ± 1.12**	**150.73**	**1.368-1.874**	**4.23**	**<0.001**
		P vs. T	1.10 ± 1.17	150.73	0.877-1.369	0.58	0.562
	Control vs. perch	**T**	**2.69 ± 1.12**	**150.73**	**2.296-3.144**	**8.87**	**<0.001**
		P	**2.00 ± 1.12**	**150.73**	**1.711-2.343**	**6.23**	**<0.001**
		P vs. T	1.34 ± 1.17	150.73	1.074-1.676	1.87	0.064

Table S3: Estimates of linear contrasts and their *P*-values corrected for false discovery rate, comparing the number of bufadienolide compounds (NBC) and total bufadienolide quantity (TBQ) between treatments. Note that here, tadpoles from different populations were pooled together. Significant differences are highlighted in bold, a marginally non-significant difference is marked with an asterisk.

Trait	Contrasts	Estimate ± SE	df	84% CI	t-ratio	*P*
NBC	Control vs. newt	**0.89 ± 0.14**	**172.07**	**0.687 - 0.084**	**6.29**	**<0.001**
	Control vs. dragonfly	**1.10 ± 0.14**	**171.48**	**0.902 - 1.298**	**7.86**	**<0.001**
	Control vs. perch	**1.37 ± 0.14**	**171.48**	**1.169 - 1.564**	**9.76**	**<0.001**
	Newt vs. dragonfly	0.21 ± 0.14	172.07	0.016 - 0.413	1.53	0.129
	Newt vs. perch	**0.48 ± 0.14**	**172.07**	**0.283 - 0.680**	**3.42**	**0.001**
	Dragonfly vs perch*	0.27 ± 0.14	171.48	0.069 - 0.464	1.90	0.070
TBQ	Control vs. newt	**499.72 ± 190.30**	**152.36**	**231.017 - 768.418**	**2.63**	**0.011**
	Control vs. dragonfly	**541.89 ± 189.29**	**152.06**	**274.618 - 809.154**	**2.86**	**0.007**
	Control vs. perch	**1084.36 ± 189.29**	**152.06**	**817.093 - 1351.629**	**5.73**	**<0.001**
	Newt vs. dragonfly	42.17 ± 190.30	152.36	-226.532 - 310.869	0.22	0.825
	Newt vs. perch	**584.64 ± 190.30**	**152.36**	**315.942 - 853.344**	**3.07**	**0.007**
	Dragonfly vs perch	**542.47 ± 189.29**	**152.06**	**275.206 - 809.743**	**2.87**	**0.007**

Fig. S1: Predator-induced responses of toad tadpoles in the number of bufadienolide compounds (NBC, panel A) and in total bufadienolide quantity (TBQ, panel B). "C" corresponds to tadpoles in the control treatment, "N" to tadpoles exposed to chemical cues of newts, "D" to dragonflies and "P" to perch. Thick horizontal lines depict medians, boxes the interquartile range, whiskers extend to the upper and lower quartile ± 1.5 × interquartile range; open circles represent extreme data points. Letters above boxplots indicate results of pairwise comparisons based on linear contrasts corrected for false discovery rate. Different letters indicate significant differences between groups, a marginally non-significant difference (P = 0.07, see Table S3) is represented by an asterisk.

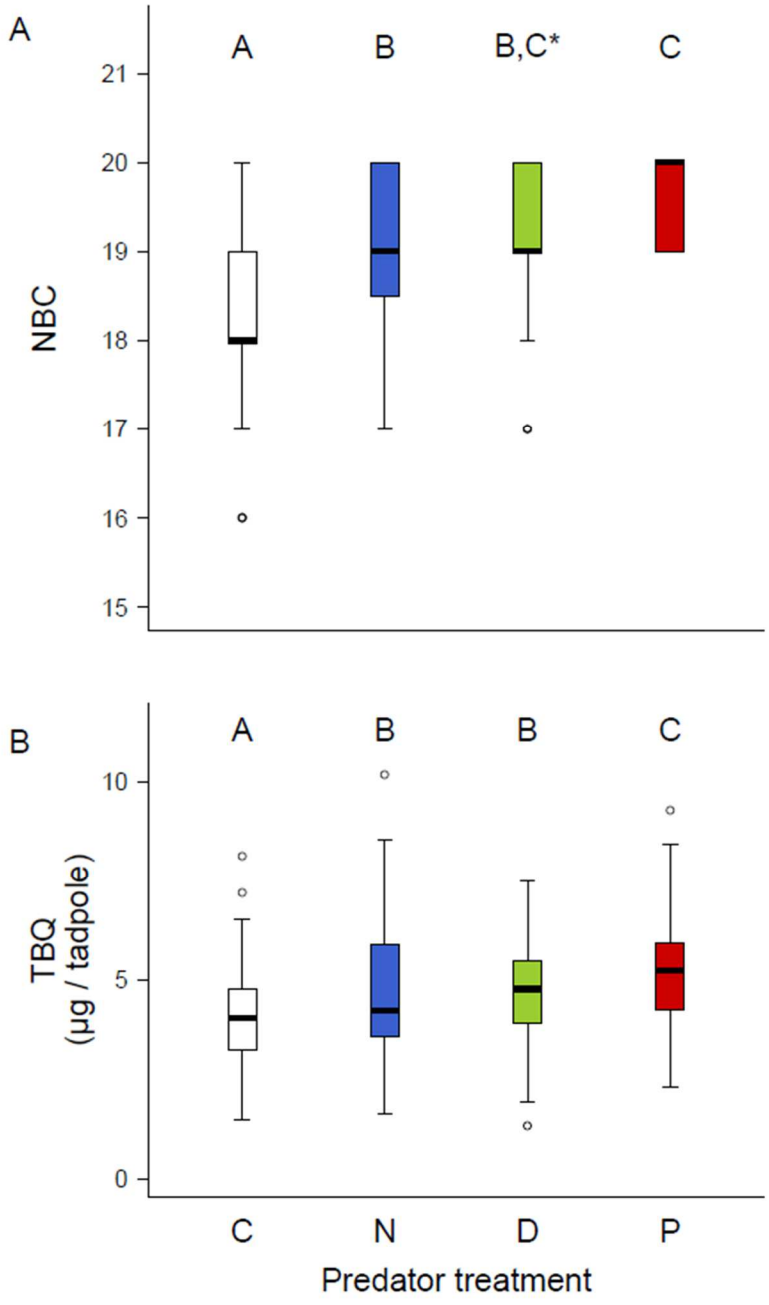
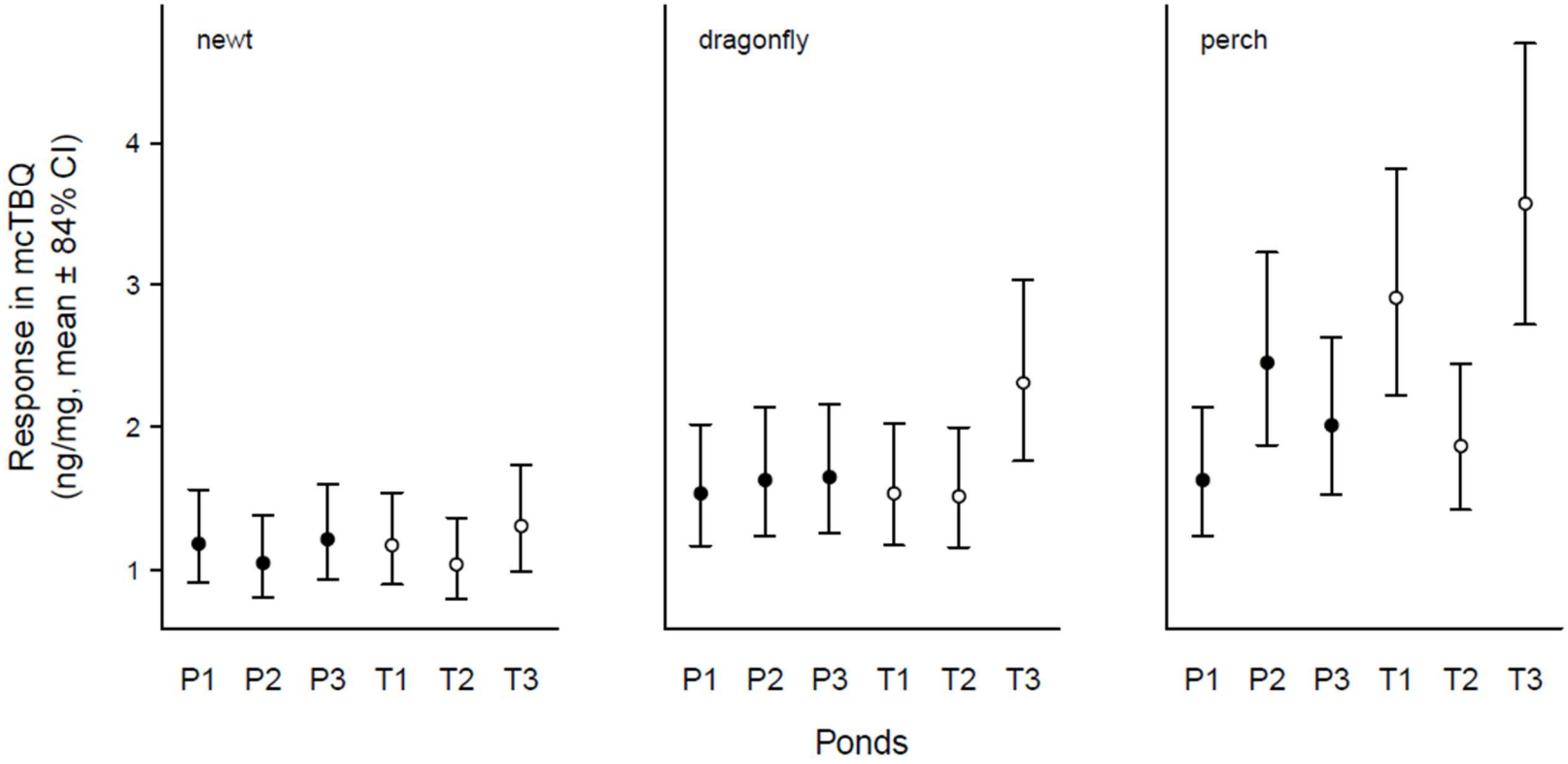


Fig. S2: The intensity of antipredator responses in mass-corrected total bufadienolide quantity (mcTBQ) in tadpoles originating from three permanent (P) and three temporary (T) ponds. Response intensity was calculated from differences in toxin production between the control and each predator treatment within each population using linear contrasts calculated from LMMs. Panel A depicts responses to newts, panel B to dragonflies, and panel C to perch. Means and 84 % confidence intervals (CI) are given.



Further scientific publications

1. **Üveges, B.**, Halpern, B., Péchy, T., Posta, J. and Komlósi, I. (2012): Characteristics and heritability analysis of the head scales of the Hungarian meadow viper (*Vipera ursinii rakosiensis*, Méhely 1893). *Amphibia-Reptilia* 33: 393-400 (Q2, IF=1,396)
2. Mizsei, E. & **Üveges, B.** (2012): Novel defensive behaviours in both sexes of *Vipera ursinii graeca* (Serpentes: Viperidae). *Herpetology Notes* 5: 481-483 (Q3)
3. Mizsei, E., **Üveges, B.**, Vági, B., Szabolcs, M., Lengyel, Sz., Pfliegler, W.P., Nagy, Z.T., Tóth, J.P. (2016): Species distribution modelling leads to the discovery of new populations of one of the least known European snakes, *Vipera ursinii graeca* in Albania. *Amphibia-Reptilia* 37: 55-68 (Q1, IF=1,396)
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