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Toxin depletion has no effect on antipredator responses in common toad (*Bufo bufo*) tadpoles

ANIKÓ KURALI*, KATALIN PÁSZTOR, ATTILA HETTYEY and ZOLTÁN TÓTH

Lendület Evolutionary Ecology Research Group, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman Ottó út 15, H-1022, Budapest, Hungary

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Antipredator responses often involve changes in several phenotypic traits and these changes interactively influence fitness. However, gaining insight into how the overall fitness effect of the overall response comes about is notoriously difficult. One promising avenue is to manipulate a single defensive trait and observe how that modifies fitness as well as the expression of other inducible responses. In chemically-defended animals, toxins are likely to be costly to produce but it is still unknown how their depletion influences other characteristics. In the present study, we artificially depleted bufadienolide toxin stores in common toad (*Bufo bufo*) tadpoles, and assessed the effect of this with respect to the interaction with predator presence and limited food availability. We found that toxin depletion in tadpoles did not significantly affect any of the measured life-history traits. Tadpoles in the predator treatment exhibited an elevated development rate, although this was only apparent when food availability was limited. Also, body mass at metamorphosis was lower in tadpoles exposed to chemical cues indicating a predation threat and when food availability was limited. These results provide evidence that, in larval common toads, the expression of inducible defences may incur fitness costs, whereas chemical defences are either expressed constitutively or, if inducible, elevated toxin production has negligible costs. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **00**, 000–000.

KEYWORDS: amphibians – chemical defence – developmental plasticity – fitness cost – trade-offs.

INTRODUCTION

Variation in phenotypic traits, including those affecting fitness, may emerge as a result of individual responses to environmental variability. Phenotypic plasticity is favoured by natural selection in populations that inhabit irregularly varying environments, and can be regarded as adaptive when the induced phenotypes confer high relative fitness in the inducing environments (Agrawal, 2001; Pigliucci, 2005; Ghalambor *et al.*, 2007). Inducible defences are plastic responses that evolve in populations facing temporal or spatial variation in predation risk (Auld & Relyea, 2011) and can take the form of morphological (Kusch, 1993; DeWitt, 1998; Ab, Herczeg & Merilä, 2016), developmental (Van Buskirk & Schmidt, 2000), behavioural (Peluc *et al.*, 2008; Green, 2009) or chemical responses (Kubanek *et al.*, 2002; Kicklighter, 2012). In predator-rich environments, induced phenotypes typically have higher survival rates than the non-induced forms; however, these phenotypes should have lower fitness in predator-free environments as a result of costs related to the production or maintenance of the induced phenotypic changes (Lively, 1986; Clark & Harvell, 1992). There is abundant empirical evidence for the presence of costs and constraints of induced defences in various animal taxa, manifesting in resource-mediated trade-offs between the defensive investment and other biological functions, such as growth or reproduction (Berenbaum & Zangerl, 1994; Tollrian & Harvell, 1999; Fordyce, Nice & Shapiro, 2006; Hammill, Rogers & Beckerman, 2008).

Amphibians exhibit plastic responses to predators during larval development in many life-history traits, such as behaviour, morphology, growth rate or developmental rate (Skelly & Werner, 1990; Relyea & Werner, 1999; Lardner, 2000; Relyea, 2004), which may in turn incur costs, including reduced survival (McCollum & Buskirk, 1996; Van Buskirk, 2000; Relyea, 2002; Steiner & Van Buskirk, 2007; Hettyey *et al.*, 2011; Ferrari *et al.*, 2015).

*Corresponding author. E-mail: kurali.aniko@agrar.mta.hu

However, in species with complex life-history such as amphibians, costs emerging as a result of the expression of inducible defences may be difficult to quantify because the associated costs do not necessarily appear simultaneously with the defensive response (Scheiner & Berrigan, 1998; Tollrian & Harvell, 1999; Van Buskirk & Saxer, 2001; Benard, 2004; Steiner, 2007). For example, predation risk experienced at the larval stage can affect post-metamorphic morphology (Van Buskirk, 2000; Van Buskirk & Saxer, 2001) or chemical defences in metamorphs (Benard & Fordyce, 2003; Hagman *et al.*, 2009) and may reduce fitness in later life stages (Smith, 1987; Semlitsch, Scott & Pechmann, 1988).

Apart from the presence and abundance of potential predators, further stress factors, such as resource availability, may also regulate the expression of different forms of inducible defences (Alford & Harris, 1988; Newman, 1994, 1998; Laurila, Kujasalo & Ranta, 1998; Winkler & Van Buskirk, 2012). According to the optimal allocation hypothesis, individuals are only able to allocate sufficient amounts of resources into all traits that are expressed at higher levels as parts of an induced response to predation threat when conditions are benign, although further trade-offs, such as genetic correlations, may limit the expression of plastic traits even in resource-abundant environments (Scheiner, Caplan & Lyman, 1991; DeWitt, Sih & Wilson, 1998; Auld, Agrawal & Relyea, 2010). If the cost of a single antipredator response could be experimentally manipulated, then it would be possible to examine how increased investment into one trait modifies the expression of other inducible life-history traits and affects fitness trade-offs associated with these traits. Addressing such questions would help to better understand the underlying evolutionary mechanisms maintaining phenotypic variation in nature. However, as a result of methodological difficulties related to the separate induction of one of a typically co-induced suite of plastic traits, this scenario has been rarely investigated in previous empirical studies of phenotypic plasticity.

In the present study, we investigated how the manipulation of toxin stores modifies the expression of predator-induced life-history responses and affects fitness trade-offs associated with these traits during the larval development of common toad (*Bufo bufo*, Linnaeus, 1758) tadpoles. Bufonids use toxins for chemical defence against predators in all ontogenetic stages (Clarke, 1997; Benard & Fordyce, 2003; Hagman *et al.*, 2009; Hayes *et al.*, 2009; Crossland *et al.*, 2012) and tadpoles of the common toad produce their toxins themselves (B. Üveges, G. Fera, Á. Móricz, D. Krüzselyi, A. Hettyey, unpubl. data). By measuring the length of larval development, body mass at metamorphosis, and larval survival, which are life-history traits known to correlate with individual fitness in amphibians (Smith, 1987; Semlitsch *et al.*, 1988; Altwegg & Reyer, 2003), we also tested for potential costs of induced defences. In chemically-defended animals, the depletion of toxin stores can be expected to influence other defensive characteristics both as a result of the energetic cost of replenishment and also because manipulated individuals are forced to rely on other defensive traits. It is still not known whether common toads are able to adjust their toxin production or whether this trait is expressed constitutively during larval stages (Hettyey, Tóth & Van Buskirk, 2014). Nevertheless, the design of the present study allowed us to specifically evaluate the costs of toxin store depletion in larval common toads and assess its effects on the expression of predator-induced defences when food availability was either *ad libitum* or limited.

We predicted that tadpoles whose toxin stores were depleted would take longer to metamorphose, have a lower body mass at metamorphosis or exhibit decreased survival rates compared to their conspecifics whose toxin stores were not manipulated, especially so in the presence of cues indicating a predation threat and when food availability is limited. This would imply that, in *B. bufo* tadpoles, the rate of toxin production is plastic, they mainly rely on their defensive toxins when facing predation threat, and there is a cost to enhanced toxin synthesis. Alternatively, tadpoles with depleted toxin stores may enhance their investment into other forms of antipredator defences and speed up their development and growth to escape from the aquatic environment and reach a size refuge in the presence of predators, whereas low food availability was again expected to be a limiting condition. This result would suggest that tadpoles can perceive the quantity of toxins they release, although the rate of toxin production is fixed or an enhanced toxin production carries low costs.

MATERIAL AND METHODS

Study species

The common toad is one of the most widespread amphibian species in Europe (Gasc *et al.*, 1997), which uses a large variety of water bodies, ranging from small puddles to permanent lakes, as breeding sites (Arnold & Ovenden, 2002). Because of the resulting high variability of their aquatic habitat, tadpoles are exposed to widely varying abundances of predators, competitors, and pathogens, and therefore can be expected to express plastic phenotypic responses (Via & Lande, 1985). Plasticity in response to predation risk is known to be present in some life-history traits of common toad tadpoles, although

responses often appear to be relatively weak compared to those of ranid tadpoles (Laurila, Kujasalo & Ranta, 1997; Laurila *et al.*, 1998; Lardner, 2000; but see also Richter-Boix, Llorente & Montori, 2007; Van Buskirk, 2009; Nunes *et al.*, 2014). The relative weakness of the responses may originate from individuals' reliance on chemical defences, although experimental evidence supporting this hypothesis is still lacking. Most bufonid species are known to contain bufadienolides (cardiotoxic steroids), including adults, tadpoles, and eggs (Mebs *et al.*, 2007; Hayes *et al.*, 2009; Sciani *et al.*, 2013) and common toad tadpoles produce several bufadienolide compounds from an early tadpole stage on (Bókony *et al.*, 2016; B. Üveges, G. Fera, Á. Móricz, D. Krüzselyi, A. Hettyey, unpubl. data). Hence, tadpoles of this species are ideal for studying energy allocation and trade-offs between phenotypic traits, including toxin production, in association with inducible anti-predator responses.

Animal collection and husbandry

We collected eight pairs of the common toad from Lake Garancsi (47°37′25″N, 18°48′27″E) located in the Pilis hills, Hungary, during the first 2 weeks of March 2013. Pairs spawned in separate 120-L plastic boxes, and the deposited egg strings were kept separately until the embryos hatched. Two days after hatchlings reached the free-swimming state, we placed the selected tadpoles (see below) individually into 2-L rearing containers filled with 0.7 L of reconstituted soft water (RSW) (APHA, 1985) and containing three or four dry beech leaves for shelter. Ambient temperature was set to 17 °C at the beginning and was allowed to gradually increase to 22 °C by the end of the experiment. Lighting was set to a 11 : 13 h dark/light cycle. Tadpoles were fed daily with a finely ground 4 : 1 mixture of rabbit chow and fish flakes. We performed complete water change in the rearing containers every 3 days.

To obtain predator cues, we collected 10 smooth newt [*Lissostriton vulgaris* (Linnaeus, 1758)] males from three ponds (47°42′27″N, 19°02′24″E; 47°42′42″N, 19°02′40″E; 47°42′48″N, 19°02′25″E) located in the Pilis hills in early spring, and kept them individually in 5-L covered plastic boxes filled with 2 L of RSW. Boxes contained a clay pot serving as a hiding place and a resting surface above the water level. We fed newts sludge worms (*Tubifex tubifex*) *ad libitum* and changed all water every day.

Experimental design

We used a full factorial randomized block design with four main factors: presence or absence of predator cues, low or high food level, and hormonal treatment (administration of norepinephrine or handling alone) applied once or thrice. We haphazardly selected 80 free-swimming tadpoles from each of the eight families and distributed them across the 16 treatment combinations, resulting in a total number of 640 tadpoles. During the set-up of the experiment, we improperly assigned three tadpoles, resulting in one extra individual in three treatment combinations and one tadpole less in another three groups. We presume that this error had a negligible effect on our results as a result of the high number of individuals at each treatment combination level. We arranged the individually-housed animals into five blocks along a known vertical temperature gradient present in our laboratory. Predator cues consisted of a homogenate of ground conspecifics (100 mL of RSW containing approximately 1.92 g of tadpoles homogenized live using a hand blender) mixed with 30 L of predator water taken from captive smooth newts (see above), simulating the presence of dangerous predators. Chemoreception is one of the most effective sensory modalities for detecting predators in the aquatic environment (Tollrian & Harvell, 1999; Schoeppner & Relyea, 2009; Hettyey *et al.*, 2010) and tadpoles react strongly to chemical cues from the simultaneous presence of cues from injured conspecifics and predators (Hagman *et al.*, 2009; Schoeppner & Relyea, 2009; Hettyey *et al.*, 2015). We added 25 mL of the above suspension every day to the rearing containers assigned to the predator treatment, whereas the same amount of RSW was added to the other rearing containers.

We also investigated the effects of food availability on the measured phenotypic responses. In the high food level treatment, individuals received *ad libitum* food (calculated as 9% of the actual tadpole body mass and resulting in some food always remaining until the next feeding occasion), whereas, in the low food level treatment, tadpoles received one-third of that amount (approximately 3% of tadpole mass). These relative amounts were raised to 12% and subsequently 4% as the development of tadpoles progressed. We measured five tadpoles from each treatment combination every 9 days to adjust the relative amounts of food to the actual body mass.

To manipulate the degree of investment into toxin production, we applied *in vivo* hormonal stimulation to deplete toxin stores present in the skin of tadpoles. *Sensu* Maag, Gehrer & Woodhams (2012), we placed tadpoles in the norepinephrine treatment group into a 3-mL, 100 μM norepinephrine-bitartrate (CAS 3414-63-9; Sigma-Aldrich) solution for 15 min. To wash off norepinephrine from the skin, we subsequently transferred individuals into a bin filled with 700 mL of RSW for 1 min and finally placed them

back into their rearing containers. Similar methods have been successfully used in both *in vitro* (Benson & Hadley, 1969; Castillo & Orce, 1997) and *in vivo* (Dockray, 1975; Rollins-Smith, 2005; Quagliata *et al.*, 2008; Giuliani & Rinaldi, 2010) studies on anurans, and also specifically on *B. bufo* tadpoles for inducing skin peptide release (Maag *et al.*, 2012). In the treatment group receiving only handling, we placed tadpoles into 3 mL of RSW and otherwise handled them the same way as described above. We applied these procedures at two frequencies: half of the tadpoles were treated three times (on days 8, 16 and 24), whereas the other half were treated only once during the experiment (on day 16).

When tadpoles approached metamorphosis, we monitored containers twice daily. Upon finding a metamorph (i.e. when at least one forelimb emerged – developmental stage 42; Gosner, 1960), we recorded it as a survivor, noted the time to metamorphosis, and measured its body mass (nearest mg). Subsequently, we moved metamorphs into large containers with some water and leaves, where they were kept until release at their parents' site of collection.

Statistical analysis

We used linear mixed-effects models to assess the effect of the applied treatments on the length of larval development and on body mass at metamorphosis and generalized linear mixed-effects models with a binomial error distribution were used to analyze the effect of treatments on larval survival. In all models, we included food level, predator treatment, administration of norepinephrine, and frequency of stimulation, together with their interactions as fixed factors, and block as a random factor. Analyses were performed using the 'glmer' and 'lmer' function of the 'lme4' package (Bates *et al.*, 2015) in R 3.2.2. (R Core Team, 2015). For model simplification, we entered all possible interactions of the investigated predictors into full models and used a backward procedure dropping the predictor with the highest *P*-value in each step, retaining only statistically significant effects ($P \leq 0.05$) in the final models (Grafen & Hails, 2002; Engqvist, 2005). Requirements of the fitted models were checked by plot diagnosis. All tests were two-tailed with alpha set to 0.05.

RESULTS

Length of larval development

We found no significant effect of the administration of norepinephrine, the frequency of stimulation or their interaction with each other and any other predictor on time to reach metamorphosis (Table 1). This suggests that the depletion of toxin stores and stress originating from the handling procedure did not influence the developmental time of tadpoles or the effect of other treatments on this response variable. However, the interaction between food availability and presence of predator cues was found to be significant (Fig. 1, Table 1). When food was limited, tadpoles reared in the presence of predator cues metamorphosed earlier (mean ± SE: 35.32 ± 0.23 days) than those kept in the absence of such cues (37.42 ± 0.22 days), whereas, when food was provided *ad libitum*, larval development was the shortest and its length did not depend on the applied predator-cue treatment (presence of predator cues: 32.45 ± 0.30 days; absence of predator cues: 32.40 ± 0.23 days).

Body mass at metamorphosis

Body mass was not affected by the administration of norepinephrine, frequency of stimulation or their interaction with each other, although we found a marginally nonsignificant effect of norepinephrine treatment in interaction with the presence of predator cues (Table 1). Tadpoles tended to have smaller body mass in the absence of predator cues when they were treated with norepinephrine compared to those that received only handling, whereas individuals in the norepinephrine and handling treatment groups had a similar body mass in the presence of predator cues. On the other hand, food availability and presence of predator cues significantly affected the body mass of tadpoles in interaction with each other (Fig. 2, Table 1). Individuals had a smaller body mass when food was limited compared to the *ad libitum* treatment group both in the presence (limited food: 164.32 ± 2.27 mg, *ad libitum* food: 205.06 ± 4.13 mg) and absence of predator cues (limited food: 249.68 ± 3.26 mg; *ad libitum* food: 305.47 ± 4.04 mg), whereas food limitation had a weaker effect in the presence of predators (Fig. 2).

Larval survival

The administration of norepinephrine and the frequency of stimulation had no significant effect on larval survival, either by themselves or in interaction with each other and any other predictors (Table 1). However, the interaction of food availability and the presence of predator cues affected tadpole survival (Fig. 3, Table 1). Individuals that received food *ad libitum* had a higher survival rate in the absence of predator cues (154 out of 160) than in their presence (130 out of 160), whereas individuals that received a limited amount of food survived at a similar rate until metamorphosis, irrespective of the presence or

Table 1. Test statistics and significance of the investigated predictors from the fitted models on length of larval development, body mass at metamorphosis, and larval survival

Dependent variable	Predictor(s)	χ^2	d.f.	*P*
Length of larval development	**Predator**	**< 0.01**	**1**	**0.976**
	Food level	**323.21**	**1**	**< 0.001**
	Predator × Food level	**25.66**	**1**	**< 0.001**
	NE	0.79	1	0.373
	Freq	0.40	1	0.528
	NE × Food	1.29	1	0.257
	NE × Predator	< 0.01	1	0.974
	Freq × Food	0.67	1	0.413
	Freq × Predator	0.26	1	0.611
	NE × Freq	0.27	1	0.604
	NE × Freq × Predator	1.12	1	0.290
	NE × Freq × Food	1.25	1	0.264
	Freq × Predator × Food	0.67	1	0.412
	NE × Predator × Food	0.53	1	0.468
	NE × Freq × Predator × Food	1.68	1	0.194
Body mass at metamorphosis	**Predator**	**467.79**	**1**	**< 0.001**
	Food	**155.87**	**1**	**< 0.001**
	Predator × Food	**5.47**	**1**	**0.019**
	NE	2.07	1	0.150
	Freq	0.18	1	0.669
	NE × Food	0.31	1	0.580
	NE × Predator	3.77	1	0.052
	Freq × Food	0.70	1	0.404
	Freq × Predator	0.13	1	0.720
	NE × Freq	0.52	1	0.470
	NE × Freq × Predator	2.05	1	0.152
	NE × Freq × Food	0.66	1	0.417
	Freq × Predator × Food	0.23	1	0.632
	NE × Predator × Food	0.16	1	0.693
	NE × Freq × Predator × Food	0.13	1	0.722
Larval survival	**Predator**	**14.77**	**1**	**< 0.001**
	Food	**2.00**	**1**	**0.157**
	Predator × Food	**7.90**	**1**	**0.005**
	NE	1.22	1	0.270
	Freq	0.27	1	0.602
	NE × Food	0.03	1	0.863
	NE × Predator	0.04	1	0.846
	Freq × Food	0.24	1	0.624
	Freq × Predator	0.04	1	0.851
	NE × Freq	< 0.01	1	0.956
	NE × Freq × Predator	0.28	1	0.598
	NE × Freq × Food	1.45	1	0.229
	Freq × Predator × Food	0.73	1	0.392
	NE × Predator × Food	1.04	1	0.308
	NE × Freq × Predator × Food	0.19	1	0.664

Wald χ^2-tests were applied for calculating *P*-values in all models using the 'Anova' function of the 'car' package (Fox & Weisberg, 2011). Final models are shown in bold; test statistics and *P*-values corresponding to the removed variables were obtained by adding them one by one to the final models. NE, norepinephrine treatment; Freq: frequency of stimulation; Predator: presence of predator cues; Food: food level.

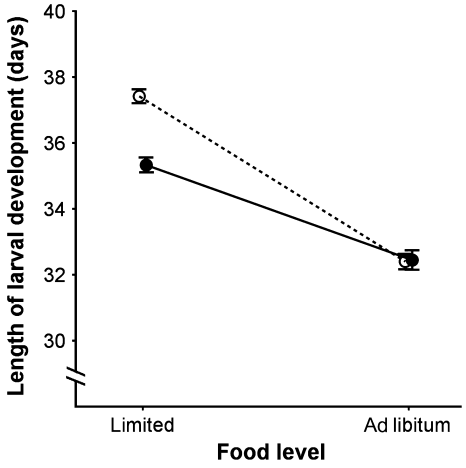

Figure 1. Effects of food restriction and simulated predator presence on the length of larval development (mean ± SE). Solid lines and filled symbols refer to the presence of predator cues; dotted lines and open symbols represent treatments without predator cues.

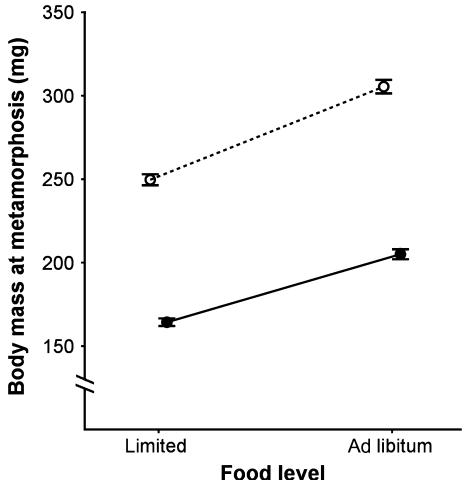

Figure 2. Effects of food restriction and simulated predator presence (mean ± SE) on body mass at metamorphosis. Solid lines and filled symbols refer to the presence of predator cues, whereas dotted lines and open symbols to the absence of predator cues.

absence of predator cues (147 out of 159 and 149 out of 161, respectively).

DISCUSSION

In the present study, we investigated how the experimental manipulation of toxin stores affected the inducible anti-predatory responses of common toad tadpoles when the presence of predator cues and food availability varied in the larval environment. We

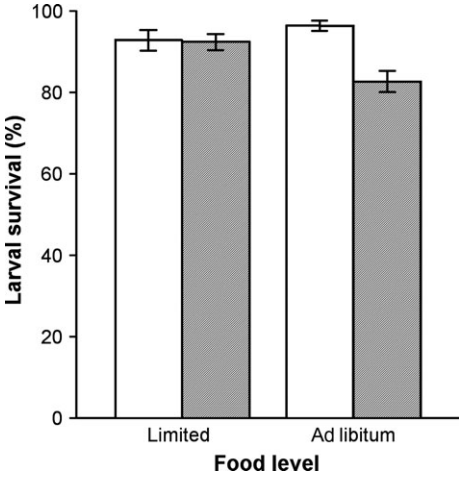

Figure 3. Proportions of tadpoles surviving until metamorphosis in the *ad libitum* or limited food groups and in the presence or absence of predator cues. Mean ± SE values (calculated from the percentage of surviving individuals in each treatment combination in each block) are depicted. Filled columns refer to the presence of predator cues; white columns represent treatments without predator cues.

found that tadpoles whose toxin stores were depleted did not take longer to metamorphose, did not have lower body mass at metamorphosis, and did not exhibit decreased survival rates compared to their conspecifics whose toxin stores were not manipulated. However, we observed that all measured traits were influenced by the interaction of food availability and predator presence. The lack of significant direct and interactive effects involving toxin store depletion and predation risk or food availability on the measured life-history traits suggests that toxin store depletion exerts no effect on the existing energetic trade-offs in the examined predator-induced responses in this species.

Administration of norepinephrine and the frequency of stimulation did not influence the measured life-history traits significantly, either on their own or in interaction with other predictors, although we found a trend for decreased body mass when tadpoles were treated with norepinephrine in the presence of predator cues. One potential explanation for the lack of measurable costs may be that the applied hormonal stimulation did not induce toxin release. However, we consider this unlikely because the applied method had been successfully used for similar purposes in a previous study (Maag *et al.*, 2012). Moreover, in an additional experiment involving the same norepinephrine treatment, we observed the successful induction of toxin release and subsequent gradual replenishment in common toad tadpoles (A. Kurali,

K. Pásztor, A. Hettyey, Z. Tóth, unpubl. data). Another possible explanation is that the toxin stores were depleted as a result of norepinephrine treatment, although toxin production lacks a plastic component. Such a constitutive rate of toxin production may be favoured by natural selection if predators are continuously present in the larval habitat, or if individuals produce toxins not only against predators, but also against pathogens or competitors. This would also help to explain why we did not find any support for the optimal allocation theory in association with toxin production and other anti-predator responses in the present study. Finally, we may not have detected costs of an induced elevated rate of toxin production if the synthesis of bufadienolides bears low costs in larval *B. bufo*, at least measured in body mass, development rate and survival, or if the production costs of these toxins are negligible compared to the maintenance cost of the synthesizing and secreting apparatus. Additionally, the lack of any effect of treatment with norepinephrine and of its administration frequency on the measured traits implies that *B. bufo* tadpoles do not perceive the quantity of toxins they release, because, otherwise, we would have expected stronger induced responses in other defensive traits in tadpoles with depleted toxin stores than in tadpoles well-defended by toxins.

Bufonid tadpoles have been found to react with an altered life-history to the simulated presence of predators (Lardner, 2000; Hagman *et al.*, 2009; but see also Benard & Fordyce, 2003) similar to larvae of many other anuran species. We showed that the length of larval development was unaffected by predation risk when food was available *ad libitum*, which suggests that, if resources are sufficiently abundant, tadpoles may be able to maintain maximal developmental rate even if feeding activity is lowered in the presence of predator cues (Abrams & Rowe, 1996; Laurila *et al.*, 1998; but see Steiner, 2007; Bennett, Pereira & Murray, 2013). On the other hand, tadpoles developed faster in the presence than in the absence of predator cues when food was scarce, and thus exhibited induced anti-predator response in the developmental rate. The latter result aligns with predictions of theoretical models (Wilbur & Collins, 1973; Abrams & Rowe, 1996) and the results of similar empirical studies (Skelly & Werner, 1990; Laurila *et al.*, 1998; Chivers *et al.*, 1999; Barry, 2015) (i.e. when predators are abundant in the aquatic environment, tadpoles are expected to escape high predation risk in the aquatic environment by metamorphosing earlier). This plastic response appeared to bear costs in terms of lowered body mass of metamorphs, which is in turn associated with a reduced fitness in later life stages (Smith, 1987; Semlitsch *et al.*, 1988; Altwegg & Reyer, 2003). Moreover, at low resource availability, we observed extended larval development also in the absence of chemical cues indicating a predation threat, supporting the hypothesis that development rate directly depends on resource availability and, thus, speeding up development leads to higher demands and is thereby costly (Skelly & Werner, 1990; Steiner, 2007).

Body mass at metamorphosis was lower in the presence of predator cues and when food availability was limited, similar to the findings of previous studies (Skelly, 1995; Anholt, Werner & Skelly, 2000; Relyea, 2002). The negative effect of food limitation on body mass may be self-evident, whereas the reduced body size when raised in the presence of predators may be interpreted as a cost of accelerated development or lowered activity (Skelly & Werner, 1990; Lardner, 2000; Relyea & Auld, 2005). However, when nutritional resources were unlimited, exposure to predators still negatively affected body mass but not the length of larval development. Thus, the lowered body mass in the presence of predators might have been a result of lowered activity rather than a trade-off between body mass and development rate (for similar results, see Skelly, 1995; Anholt *et al.*, 2000). The effect of predator presence on body mass was larger in the high food treatment, which corresponds to results of previous studies (Steiner, 2007). However, some studies observed opposite trends, where *ad libitum* food increased the size of tadpoles in the presence of predator cues (Bennett *et al.*, 2013), whereas others found no interaction between food and predator manipulations (Skelly & Werner, 1990; LaFiandra & Babbitt, 2004). The discrepancy among experimental results suggests that increased body size may be an adaptive defensive response in some species or when facing some types of (e.g. gape-limited) predators (Kishida & Nishimura, 2005; Urban, 2007), whereas, in *B. bufo* tadpoles, the allocation of limited resources may be directed into other defensive traits, such as a reduced developmental time.

Survival costs of anti-predator defences are expected to be rare (Werner & Anholt, 1993; Van Buskirk, 2000), although they have been documented previously in larval amphibians (McCollum & Buskirk, 1996; Relyea & Hoverman, 2003; Hettyey *et al.*, 2011). The underlying mechanism may involve behavioural adaptations to the presence of predators resulting in reduced swimming and feeding activity of tadpoles (Lawler, 1989; Skelly & Werner, 1990; Marquis, Saglio & Neveu, 2004), which, in turn, can lead to lowered amounts of food intake. Consequently, survival costs are expected to be higher when food is scarce. By contrast, we found that survival was lower in tadpoles raised in predator presence when food was provided *ad libitum* but not when food availability was limited. One possible explanation for this finding is the

increased presence of leftovers as a result of the reduced feeding activity of tadpoles in the *ad libitum* food treatment group when predator cues were present, which may have served as a substrate for fungi or bacteria, causing mortality in tadpoles (Russo & Yanong, 2006; Roberts & Palmeiro, 2008). Nonetheless, this additional stress did not appear to negatively affect those individuals that reached metamorphosis in the *ad libitum* food treatment group because they had a greater body mass at metamorphosis and the shortest length of larval development both in the presence and absence of predator cues in their environment compared to those raised in the limited food treatment group.

In conclusion, we did not detect significant fitness costs of toxin store depletion in *B. bufo* tadpoles, although we found altered life-history traits in response to food limitation and simulated predator presence. Our findings suggest that common toad tadpoles exhibit an adaptive anti-predatory plastic response in terms of elevated development rate, although this is only apparent in a resource-limited environment and is associated with the fitness cost of having lowered body mass at metamorphosis. This relationship was, however, not affected by the experimental manipulation of toxin stores; thus, chemical defence with bufadienolides may have negligible production costs measured in body mass, development rate, and survival or may be expressed constitutively in this species. Nevertheless, the present study represents an important step towards a better understanding of how interactions of multiple environmental factors may form inducible anti-predator defences in species with a complex life history.

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REFERENCES

Ab Ghani NI, Herczeg G, Merilä J. 2016. Effects of perceived predation risk and social environment on the development of three-spined stickleback (*Gasterosteus aculeatus*) morphology. *Biological Journal of the Linnean Society***118:** 520–535.

Abrams PA, Rowe L. 1996. The effects of predation on the age and size of maturity of prey. *Evolution* **50:** 1052–1061.

Agrawal AA. 2001. Phenotypic plasticity in the interactions and evolution of species. *Science* **294:** 321–326.

Alford RA, Harris RN. 1988. Effects of larval growth history on anuran metamorphosis. *American Naturalist* **131:** 91.

Altwegg R, Reyer HU. 2003. Patterns of natural selection on size at metamorphosis in water frogs. *Evolution* **57:** 872–882.

Anholt BR, Werner E, Skelly DK. 2000. Effect of food and predators on the activity of four larval ranid frogs. *Ecology* **81:** 3509–3521.

APHA. 1985. *Standard methods for the examination of wastewater*, 16th edn. Washington, DC: American Public Health Association.

Arnold N, Ovenden D. 2002. *A field guide to the reptiles and amphibians of Britain and Europe*. New York, NY: Harper Collins Publishers.

Auld J, Relyea R. 2011. Adaptive plasticity in predator-induced defenses in a common freshwater snail: altered selection and mode of predation due to prey phenotype. *Evolutionary Ecology* **25:** 189–202.

Auld JR, Agrawal AA, Relyea RA. 2010. Re-evaluating the costs and limits of adaptive phenotypic plasticity. *Proceedings of the Royal Society of London Series B, Biological Sciences* **277:** 503–511.

Barry MJ. 2015. Effects of resource distribution on the cost of predator avoidance behaviour in tadpoles. *Hydrobiologia* **758:** 99–105.

Bates D, Maechler M, Bolker B, Walker S. 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* **67:** 1–48.

Benard MF. 2004. Predator-induced phenotypic plasticity in organisms with complex life histories. *Annual Review of Ecology, Evolution, and Systematics* **35:** 651–673.

Benard MF, Fordyce JA. 2003. Are induced defenses costly? Consequences of predator-induced defenses in Western toads, *Bufo boreas*. *Ecology* **84:** 68–78.

Bennett AM, Pereira D, Murray DL. 2013. Investment into defensive traits by anuran prey (*Lithobates pipiens*) is mediated by the starvation-predation risk trade-off. *PLoS ONE* **8:** e82344.

Benson BJ, Hadley ME. 1969. In vitro characterization of adrenergic receptors controlling skin gland secretion in two

anurans *Rana pipiens* and *Xenopus laevis*. *Comparative Biochemistry and Physiology* **30:** 857–864.

Berenbaum MR, Zangerl AR. 1994. Costs of inducible defense: protein limitation, growth, and detoxification in Parsnip webworms. *Ecology* **75:** 2311.

Bókony V, Móricz ÁM, Tóth Z, Gál Z, Kurali A, Mikó Z, Pásztor K, Szederkényi M, Tóth Z, Ujszegi J, Üveges B, Krüzselyi D, Capon RJ, Hoi H, Hettyey A. 2016. Variation in Chemical Defense Among Natural Populations of Common Toad, Bufo bufo, Tadpoles: the Role of Environmental Factors. *Journal of Chemical Ecology* **42:** 329–338.

Castillo GA, Orce GG. 1997. Response of frog and toad skin to norepinephrine. *Comparative Biochemistry and Physiology Part A: Physiology* **118:** 1145–1150.

Chivers DP, Kiesecker JM, Marco A, Wildy EL, Blaustein AR. 1999. Shifts in life history as a response to predation in western toads (*Bufo boreas*). *Journal of Chemical Ecology* **25:** 2455–2463.

Clark CW, Harvell CD. 1992. Inducible defenses and the allocation of resources: a minimal model. *American Naturalist* **139:** 521–539.

Clarke BT. 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. *Biological Reviews of the Cambridge Philosophical Society* **72:** 365–379.

Crossland MR, Haramura T, Salim AA, Capon RJ, Shine R. 2012. Exploiting intraspecific competitive mechanisms to control invasive cane toads (*Rhinella marina*). *Proceedings of the Royal Society of London Series B, Biological Sciences* **279:** 3436–3442.

DeWitt TJ. 1998. Costs and limits of phenotypic plasticity: tests with predator-induced morphology and life history in a freshwater snail. *Journal of Evolutionary Biology* **11:** 465–480.

DeWitt TJ, Sih A, Wilson DS. 1998. Costs and limits of phenotypic plasticity. *Trends in Ecology & Evolution* **13:** 77–81.

Dockray G. 1975. Caerulein secretion by dermal glands in *Xenopus laevis*. *The Journal of Cell Biology* **64:** 724–733.

Engqvist L. 2005. The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Animal Behaviour* **70:** 967–971.

Ferrari MCO, Crane AL, Brown GE, Chivers DP. 2015. Getting ready for invasions: can background level of risk predict the ability of naïve prey to survive novel predators? *Scientific Reports* **5:** 8309.

Fordyce JA, Nice CC, Shapiro AM. 2006. A novel tradeoff of insect diapause affecting a sequestered chemical defense. *Oecologia* **149:** 101–106.

Fox J, Weisberg S. 2011. *An R companion to applied regression*, 2nd edn. Thousand Oaks, CA: Sage. Available at: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

Gasc JP, Cabela A, Crnobrnja-Isailovic J, Dolmen D, Grossenbacher K, Haffner P, Lescure J, Martens H, Martínez Rica JP, Maurin H, Oliveira ME, Sofianidou TS, Veith M, Zuiderwijk A, eds. 1997. Atlas of amphibians and reptiles in Europe. *Collection Patrimoines Naturels, 29*. Paris: Societas Europaea Herpetologica, Muséum National d'Histoire Naturelle & Service du Patrimoine Naturel.

Ghalambor CK, McKay JK, Carroll SP, Reznick DN. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology* **21:** 394–407.

Giuliani A, Rinaldi AC, eds. 2010. *Antimicrobial peptides*. Totowa, NJ: Humana Press.

Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16:** 183–190.

Grafen A, Hails R. 2002. *Modern statistics for the life sciences*. Oxford: Oxford University Press.

Green J. 2009. The distribution and variation of *Daphnia lumholtzi* (*Crustacea: Cladocera*) in relation to fish predation in Lake Albert, East Africa. *Journal of Zoology* **151:** 181–197.

Hagman M, Hayes RA, Capon RJ, Shine R. 2009. Alarm cues experienced by cane toad tadpoles affect post-metamorphic morphology and chemical defences. *Functional Ecology* **23:** 126–132.

Hammill E, Rogers A, Beckerman AP. 2008. Costs, benefits and the evolution of inducible defences: a case study with *Daphnia pulex*. *Journal of Evolutionary Biology* **21:** 705–715.

Hayes RA, Crossland MR, Hagman M, Capon RJ, Shine R. 2009. Ontogenetic variation in the chemical defenses of cane toads (*Bufo marinus*): toxin profiles and effects on predators. *Journal of Chemical Ecology* **35:** 391–399.

Hettyey A, Zsarnóczai S, Vincze K, Hoi H, Laurila A. 2010. Interactions between the information content of different chemical cues affect induced defences in tadpoles. *Oikos* **119:** 1814–1822.

Hettyey A, Vincze K, Zsarnóczai S, Hoi H, Laurila A. 2011. Costs and benefits of defences induced by predators differing in dangerousness. *Journal of Evolutionary Biology* **24:** 1007–1019.

Hettyey A, Tóth Z, Van Buskirk J. 2014. Inducible chemical defences in animals. *Oikos* **123:** 1025–1028.

Hettyey A, Tóth Z, Thonhauser KE, Frommen JG, Penn DJ, Van Buskirk J. 2015. The relative importance of prey-borne and predator-borne chemical cues for inducible antipredator responses in tadpoles. *Oecologia* **179:** 699–710.

Kicklighter C. 2012. Chemical defences against predators. In: Brönmark C, Hansson LA, ed. *Chemical ecology in aquatic systems*. Oxford: Oxford University Press, 236–249.

Kishida O, Nishimura K. 2005. Multiple inducible defences against multiple predators in the anuran tadpole, *Rana pirica*. *Evolutionary Ecology Research* **7:** 619–631.

Kubanek J, Whalen KE, Engel S, Kelly SR, Henkel TP, Fenical W, Pawlik JR. 2002. Multiple defensive roles for triterpene glycosides from two caribbean sponges. *Oecologia* **131:** 125–136.

Kusch J. 1993. Behavioural and morphological changes in ciliates induced by the predator *Amoeba proteus*. *Oecologia* **96:** 354–359.

LaFiandra EM, Babbitt KJ. 2004. Predator induced phenotypic plasticity in the pinewoods tree frog, Hyla femoralis: necessary cues and the cost of development. *Oecologia* **138:** 350–359.

Lardner B. 2000. Morphological and life history responses to predators in larvae of seven anurans. *Oikos* **88:** 169–180.

Laurila A, Kujasalo J, Ranta E. 1997. Different antipredator behaviour in two anuran tadpoles: effects of predator diet. *Behavioral Ecology and Sociobiology* **40:** 329–336.

Laurila A, Kujasalo J, Ranta E. 1998. Predator-induced changes in life history in two anuran tadpoles: effects of predator diet. *Oikos* **83:** 307–317.

Lawler SP. 1989. Behavioural responses to predators and predation risk in four species of larval anurans. *Animal Behaviour* **38:** 1039–1047.

Lively CM. 1986. Predator-induced shell dimorphism in the Acorn barnacle *Chthamalus anisopoma*. *Evolution* **40:** 232–242.

Maag N, Gehrer L, Woodhams DC. 2012. Sink or swim: a test of tadpole behavioral responses to predator cues and potential alarm pheromones from skin secretions. *Journal of Comparative Physiology A* **198:** 841–846.

Marquis O, Saglio P, Neveu A. 2004. Effects of predators and conspecific chemical cues on the swimming activity of *Rana temporaria* and *Bufo bufo* tadpoles. *Archiv für Hydrobiologie* **160:** 153–170.

McCollum SA, Buskirk JV. 1996. Costs and benefits of a predator-induced polyphenism in the Gray treefrog *Hyla chrysoscelis*. *Evolution* **50:** 583.

Mebs D, Wagner MG, Pogoda W, Maneyro R, Kwet A, Kauert G. 2007. Lack of bufadienolides in the skin secretion of red bellied toads, *Melanophryniscus* spp. (*Anura*, *Bufonidae*), from Uruguay. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **144:** 398–402.

Newman RA. 1994. Effects of changing density and food level on metamorphosis of a desert amphibian, *Scaphiopus couchii*. *Ecology* **75:** 1085.

Newman RA. 1998. Ecological constraints on amphibian metamorphosis: interactions of temperature and larval density with responses to changing food level. *Oecologia* **115:** 9–16.

Nunes AL, Orizaola G, Laurila A, Rebelo R. 2014. Rapid evolution of constitutive and inducible defenses against an invasive predator. *Ecology* **95:** 1520–1530.

Peluc SI, Sillett TS, Rotenberry JT, Ghalambor CK. 2008. Adaptive phenotypic plasticity in an island songbird exposed to a novel predation risk. *Behavioral Ecology* **19:** 830–835.

Pigliucci M. 2005. Evolution of phenotypic plasticity: where are we going now? *Trends in Ecology & Evolution* **20:** 481–486.

Quagliata S, Malentacchi C, Giachi F, Delfino G. 2008. Chemical skin defence in the Eastern fire-bellied toad *Bombina orientalis*: an ultrastructural approach to the mechanism of poison gland rehabilitation after discharge. *Acta Herpetologica* **3:** 139–153.

R Core Team 2015. R: a language and environment for statistical computing. Vienna:R Foundation for Statistical Computing. Available at: https://www.R-project.org

Relyea RA. 2002. Costs of phenotypic plasticity. *American Naturalist* **159:** 272–282.

Relyea RA. 2004. Fine-tuned phenotypes: tadpole plasticity under 16 combinations of predators and competitors. *Ecology* **85:** 172–179.

Relyea RA, Auld JR. 2005. Predator- and competitor-induced plasticity: how changes in foraging morphology affect phenotypic trade-offs. *Ecology* **86:** 1723–1729.

Relyea RA, Hoverman JT. 2003. The impact of larval predators and competitors on the morphology and fitness of juvenile treefrogs. *Oecologia* **134:** 596–604.

Relyea RA, Werner EE. 1999. Quantifying the relation between predator-induced behavior and growth performance in larval anurans. *Ecology* **80:** 2117–2124.

Richter-Boix A, Llorente GA, Montori A. 2007. A comparative study of predator-induced phenotype in tadpoles across a pond permanency gradient. *Hydrobiologia* **583:** 43–56.

Roberts H, Palmeiro BS. 2008. Toxicology of aquarium fish. *The Veterinary Clinics of North America. Exotic Animal Practice* **11:** 359–374, VII.

Rollins-Smith LA. 2005. Antimicrobial peptide defenses in amphibian skin. *Integrative and Comparative Biology* **45:** 137–142.

Russo J-AR, Yanong RPE. 2006. Molds in fish feeds and aflatoxins. Institute of Food and Agricultural Sciences. University of Florida Fact Sheet FA-95. Available at: http://edis.ifas.ufl.edu. Accessed 14 September 2007.

Scheiner SM, Berrigan D. 1998. The genetics of phenotypic plasticity. VIII. The cost of plasticity in *Daphnia pulex*. *Evolution* **52:** 368.

Scheiner SM, Caplan RL, Lyman RF. 1991. The genetics of phenotypic plasticity. III. Genetic correlations and fluctuating asymmetries. *Journal of Evolutionary Biology* **4:** 51–68.

Schoeppner NM, Relyea RA. 2009. Interpreting the smells of predation: how alarm cues and kairomones induce different prey defences. Unravelling the chemical cues of predation. *Functional Ecology* **23:** 1114–1121.

Sciani JM, Angeli CB, Antoniazzi MM, Jared C, Pimenta DC. 2013. Differences and similarities among parotoid macrogland secretions in South American toads: a preliminary biochemical delineation. *The Scientific World Journal* **2013:** 1–9.

Semlitsch RD, Scott DE, Pechmann JHK. 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. *Ecology* **69:** 184.

Skelly DK. 1995. A behavioral trade-off and its consequences for the distribution of pseudacris treefrog larvae. *Ecology* **76:** 150.

Skelly DK, Werner EE. 1990. Behavioral and life-historical responses of larval american toads to an odonate predator. *Ecology* **71:** 2313.

Smith DC. 1987. Adult recruitment in chorus frogs: effects of size and date at metamorphosis. *Ecology* **68:** 344.

Steiner UK. 2007. Linking antipredator behaviour, ingestion, gut evacuation and costs of predator-induced responses in tadpoles. *Animal Behaviour* **74:** 1473–1479.

Steiner UK, Van Buskirk J. 2007. Environmental stress and the costs of whole-organism phenotypic plasticity in tadpoles. *Journal of Evolutionary Biology* **21:** 97–103.

Tollrian R, Harvell CD, eds. 1999. *The ecology and evolution of inducible defenses*. Princeton, NJ: Princeton University Press.

Urban MC. 2007. The growth-predation risk trade-off under a growing gape-limited predation threat. *Ecology* **88:** 2587–2597.

Van Buskirk J. 2000. The costs of an inducible defense in anuran larvae. *Ecology* **81:** 2813–2821.

Van Buskirk J. 2009. Natural variation in morphology of larval amphibians: phenotypic plasticity in nature? *Ecological Monographs* **79:** 681–705.

Van Buskirk J, Saxer G. 2001. Delayed costs of an induced defense in tadpoles? Morphology, hopping, and development rate at metamorphosis. *Evolution* **55:** 821–829.

Van Buskirk J, Schmidt BR. 2000. Predator-induced phenotypic plasticity in larval newts: trade-offs, selection, and variation in nature. *Ecology* **81:** 3009–3028.

Via S, Lande R. 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. *Evolution* **39:** 505–522.

Werner EE, Anholt BR. 1993. Ecological consequences of the trade-off between growth and mortality rates mediated by foraging activity. *American Naturalist* **142:** 242.

Wilbur HM, Collins JP. 1973. Ecological aspects of amphibian metamorphosis: nonnormal distributions of competitive ability reflect selection for facultative metamorphosis. *Science* **182:** 1305–1314.

Winkler JD, Van Buskirk J . 2012. Influence of experimental venue on phenotype: multiple traits reveal multiple answers: venue and experimental outcome. *Functional Ecology* **26:** 513–521.