



Resource-dependent temporal changes in antipredator behavior of common toad (*Bufo bufo*) tadpoles

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Abstract

Inducible behavioral defenses against predators, and how environmental factors mediate such responses, have been the focus of behavioral ecological research for decades. However, results often remained contradictory, perhaps because the ontogenetic context was ignored. Here, we investigated how antipredator behavioral responses of common toad (*Bufo bufo*) tadpoles are affected by food limitation and how the mediated responses changed during larval development. We raised tadpoles in the presence or absence of chemical cues indicating predation risk, combined with low or high food levels, and repeatedly monitored tadpole activity and visibility. We found that the presence of cues indicating predation risk and resource availability interactively affected visibility, but not relative activity, and this interactive effect changed with time over the larval period. Visibility of tadpoles decreased with time but to a greater extent when tadpoles were exposed to cues indicating predation risk compared to control groups, and this difference was more expressed when food was limited. Activity of tadpoles also decreased during larval development but to a greater extent in case of tadpoles raised in resource-limited environment compared to the other treatments. Also, activity of tadpoles was higher when food was scarce; however, the magnitude of this effect was not influenced by the predator-cue treatment. Thus, in addition to describing the trajectories of ontogenetic changes in tadpole behavior, our study also demonstrated that responses to environmental factors, such as predation threat and food availability, vary with age.

Significance statement

Antipredator behavior is a common phenomenon in nature, and its expression is known to be influenced by the amount of available resources. How such defensive responses change with age, however, has been studied barely. By repeatedly observing common toad (*Bufo bufo*) tadpoles exposed to predators and/or food limitation, we found that tadpoles' activity and visibility decreased with age and did so to a greater extent when tadpoles were exposed to chemical cues indicating predation risk compared to control groups. Our study demonstrated how responses to environmental factors, such as predation threat and food availability, vary with age in an important amphibian model species. These results draw attention to the importance of considering age when studying behavioral responses to environmental change and provide a possible explanation for some inconsistencies in the relevant literature.

Keywords Toad · Behavioral plasticity · Feeding activity · Developmental changes · Trade-off

Introduction

Searching for food while avoiding predators is one of the crucial balancing acts in the life of a prey animal, since their

activity is governed by a trade-off between foraging and predator avoidance (Milinski and Heller 1978; Werner and Gilliam 1984; McNamara 1987; Lima and Dill 1990; Anholt and Werner 1995). According to the allocation model, prey animals with higher resource availability can invest surplus energy into antipredator defenses (Harvell 1990; Werner and Anholt 1993), while individuals in poorer condition should be more risk-prone (e.g., Milinski and Heller 1978; McNamara 1987; Anholt and Werner 1995) in order to maintain basic life functions when no investment in defense can be afforded, or suffer potentially detrimental lower energy gain associated with reduced foraging activity (e.g., Cowlshaw

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1997; for a review see Brown and Kotler 2004). In contrast to the allocation model, the defense-growth model (Myers and Bazely 1991) predicts that investment in defense should be higher at low resource availability. If growth is reduced at low resource availability, prey individuals will spend more time at vulnerable stages (Arendt 1997), hence the elevated need for induced defenses, while at high resource availability, individuals can grow quickly, allowing them to escape predation risk either by metamorphosing early or by rapidly reaching a size that provides protection from gape-limited predators (Kishida and Nishimura 2005). The growth-differentiation model (e.g., Steiner and Pfeiffer 2007) combines the previous two models. Even though investment into defense is problematic to measure directly, the expression of defensive traits is clearly a function of underlying time and resource allocation, and the plasticity of these traits is alternative estimate to the investment into antipredator defenses (Van Buskirk 2000).

Numerous amphibian species exhibit plastic responses to avoid predation during larval ontogeny with changes in their morphology, physiology, or behavior (Wells 2007), but the expression of plasticity is also constrained by inherent costs and limits (DeWitt et al. 1998) and balanced according to the energetic requirements of post-metamorphic performance and fitness (e.g., Van Buskirk 2000; Relyea 2002). Behavioral trade-offs between foraging and avoiding predators have been shown to highly depend on the availability (e.g., Skelly 1995; Laurila et al. 1998) and quality of food (e.g., Eklöv and Werner 2000; Eklöv and Halvarsson 2000), affecting growth rate and size at metamorphosis (Kupferberg 1997; Laurila et al. 1998; Winkler and Van Buskirk 2012), as well as survival and reproductive success of adults (Alford and Harris 1988; Cabrera-Guzmán et al. 2013). The behavioral phenotype of tadpoles can also be affected by ontogeny and resulting changes in body size (Werner and Anholt 1993). A large body of empirical work shows that the duration and timing of exposure to predation threat and of behavioral observations have crucial importance for experiments investigating tadpole behavior (Eklöv and Werner 2000; Hossie and Murray 2012; Wilson and Krause 2012; Touchon et al. 2013; Bateman and Fleming 2015); however, whether predation risk and resource availability mediate tadpoles' behavior interactively, but variably during ontogeny, has rarely been investigated (for an exception see Laurila et al. 1998; Bennett et al. 2013). In non-anuran prey species, previous studies showed that the importance of such environmental factors may indeed change during ontogenetic development (insects: Dangles et al. 2007; Wohlfahrt et al. 2007, fish: Werner and Hall 1988; Dahlgren and Eggleston 2000, reptiles: Roth and Johnson 2004).

In this study, we investigated how the interactive effect of predation threat and food limitation on antipredator behavior of common toad (*Bufo bufo*) tadpoles changed with time

during ontogeny. By performing repeated observations on tadpoles, we examined at various stages of larval development to what extent predation threat led to lowered feeding activity and increased concealment, which are the most common behavioral responses of anuran larvae in response to predators (Eklöv and Werner 2000; Richardson 2001; Jara and Perotti 2010). We presumed that food limitation would moderate behavioral responses to predators (e.g., Almeida et al. 2011; Nunes et al. 2014). Because foraging efficiency of tadpoles increases with age (Hensley 1993; Hentschel 1999), we predicted lowered activity in more developed tadpoles. As energetic costs arising from behavioral defenses generally decrease during development (Werner and Anholt 1993; Urban 2007b; Cressler et al. 2010), higher levels of behavioral defenses could be expected in more developed larvae. However, the type of predator used in our experiment may also strongly influence the behavior of tadpoles during the larval period (Hossie et al. 2017). Because we used chemical cues from a gape-limited predator, we also expected that behavioral defenses might diminish with age (Kishida and Nishimura 2005; Urban 2007a, 2007b) as tadpoles grow beyond the size these predators can effectively prey upon. Finally, we predicted food limitation would lead to elevated activity and enhance the costs of behavioral defenses, manifesting in decreased antipredator responses (Anholt and Werner 1995; Anholt et al. 2000; Bridges 2002).

Materials and methods

The study species

The common toad (*Bufo bufo*) is one of the most widespread anuran species in Europe (Sillero et al. 2014). It uses a wide variety of water bodies as breeding sites, ranging from ephemeral puddles to permanent lakes, which exposes larvae to widely and unpredictably varying biotic and abiotic environments during the aquatic life-stage. Tadpoles of *B. bufo* are known to produce skin toxins (Toledo and Jared 1995; Bókony et al. 2016) that provide some level of protection against predators (Reading 1990). Moreover, they react with changes in life history (i.e., enhanced development rate, see Laurila et al. 1998; Lardner 2000; Almeida et al. 2011); morphology (Van Buskirk 2009; Nunes et al. 2014); and, especially, behavior (Marquis et al. 2004; Nunes et al. 2013; but see Richter-Boix et al. 2007) to the simulated presence of predators and to its interaction with limited food availability (e.g., Laurila et al. 1998).

Collection and maintenance of animals

We collected eight pairs of *B. bufo* during early spring from a pond (47°37'26"N, 18°48'27"E) ca. 20 km to the NW of

Budapest, Hungary, and transported them to the laboratory at the Experimental Station Júliannamajor (Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences). We let individual pairs spawn in large plastic boxes and subsequently reared embryos separately according to families until hatching.

We captured 15 smooth newt (*Lissotriton vulgaris*) males using plastic funnel traps from three ponds in the Pilis-hills, Hungary (47°42'27"N, 19°02'24"E; 47°42'42"N, 19°02'40"E; 47°42'48"N, 19°02'25"E). We transported newts to the laboratory and placed them individually in 5-L plastic boxes. Each box contained 2 L of RSW and a clay pot serving as a submerged hiding place and a resting surface above the water level. We fed newts with sludge worms (*Tubifex spp.*) ad libitum and changed water every day. After termination of the experiment, all animals were released at the site of their collection.

Experimental procedures

Two days after hatchlings reached the free-swimming state (developmental stage 25; Gosner 1960), we started the experiment by haphazardly selecting 40 healthy tadpoles from each of the eight families and placing them individually into 2-L rearing dishpans. Individual housing was applied to maximize the number of statistically independent units and avoid the potential confounding effect of competition between conspecifics in chemical defense (please note that the investigation of toxin production was also part of the experimental design; see below). Rearing dishpans contained 0.7 L reconstituted soft water (RSW; APHA 1985) and three beech leaves providing shelter for tadpoles. The temperature in the laboratory varied between 17 and 22 °C, and the lighting was set to a 13:11 light/dark cycle. In order to control for temperature variation in the laboratory, we arranged the experiment in spatial blocks using a factorial randomized block design with two factors: presence vs. absence of cues indicating predation risk and low vs. high food level. Tadpoles were assigned randomly to treatments. This resulted in 80 replicates in each treatment combination and a total number of 320 tadpoles of which 312 survived until the end of the experiment. During the set-up of the experiment, we improperly assigned tadpoles from the 8 families, resulting in unbalanced numbers of offspring in the treatment combinations (ranging from 5 to 17 instead of having 10 tadpoles/family assigned to each combination). We presume that this error had a negligible effect on our results, because we still had a sufficient number of replicates for each family in each treatment combination. The study animals also took part in another experiment (Kurali et al. 2016), but tadpole behavior was not analyzed there. In Kurali et al. (2016), we addressed a fundamentally different research question, namely how artificially induced toxin depletion affected other predator-induced plastic responses and whether it manifested in fitness-related parameters in common toad tadpoles. During

the course of that experiment, we also conducted behavioral observations on tadpoles in the control group to investigate the significance of ontogenetic changes in antipredator behavior of tadpoles, the results of which we present here.

We exposed tadpoles to the presence or absence of cues indicating predation risk crossed with either low or high food level in a full factorial design. We simulated predator presence by the daily addition of 25 ml of a suspension containing ground conspecifics and water taken from captive newts into tadpoles' rearing dishpans. We prepared the suspension by homogenizing approx. 1.92 g (between 6 and 20 individuals) of *B. bufo* tadpoles in 100 mL RSW right before cue addition, and mixing this with 30 l of water taken from captive smooth newts. The rest of the suspension was used in a parallel experiment. We added the same amount of RSW to rearing dishpans assigned to the control group. Chemoreception is widely used by aquatic animals for assessing predation risk (Tollrian and Harvell 1999; Schoeppner and Relyea 2009; Hettyey et al. 2010), and tadpoles of many anurans, including our model species, are known to react to a combination of chemical cues from crushed conspecifics and from predators (Marquis et al. 2004; Hagman et al. 2009; Schoeppner and Relyea 2009; Nunes et al. 2013; Hettyey et al. 2015). We fed tadpoles daily with a finely ground 4:1 mixture of rabbit chow and fish flakes. Larvae fed ad libitum received an amount equaling 9% of their body mass, which we raised to 12% after 2 weeks and one third of that amount (3%, later 4% of tadpole mass) in the restricted food treatment. We adjusted the quantity of food to changing body mass every 9 days by measuring mass of five randomly selected tadpoles from each treatment combination. We changed water every third day.

Behavioral data were collected on six different days: 5, 9, 13, 17, 21, and 25 days after start of the experiment, eight times a day. These observations covered almost the entire larval period of common toads in our experiment, because the first individuals started to metamorphose already on day 27. We carefully approached rearing dishpans and recorded visibility and activity of tadpoles. The observation of each box lasted only a few seconds, until the given tadpole was unambiguously located and its behavior categorized. We scored an individual as "hiding" when it was hidden under leaves or "visible" when any part of its body was visible and "inactive" when it was visible but no movement was observable or "active" when we could observe movement of any part of the body. To minimize observer bias, blinded methods were used when all behavioral data were recorded and/or analyzed.

Statistical analyses

Analyses were based on restricted maximum likelihood estimation using the "glmmadmb" function of the "glmmADMB" package (Fournier et al. 2012; Skaug et al. 2013) in R 3.1.1. (R Core Team 2017). We used generalized mixed-effects

models to assess treatment effects on tadpole behavior during larval development. Visibility was computed by summing the number of occasions when tadpoles were visible from the total number of eight observations per day. Relative activity was calculated by dividing the number of occasions when tadpoles were scored active by the number of occasions when they were visible. We fitted the models with binomial error distribution in the case of visibility and with beta binomial error distribution in the case of relative activity. We entered behavioral data as dependent variables, whereas time, food availability, and predator-cue treatment were fixed factors. We also included individual identity and family as nested random factors (individual identity nested into family) and block as a crossed random factor in order to control for pseudo-replication at various levels (individual, clutch-of-origin, and spatial location, respectively; for a similar approach, see Laurila et al. 1998). We added time as a covariate to the nested random term to allow different slopes of the behavioral responses to treatments over time for each individual. In the case of visibility, we also added a crossed observation-level random factor to the model, which is a robust method of accounting for overdispersion in GLMMs (e.g., Browne et al. 2005; Harrison 2014); this was unnecessary in the case of relative activity, where we found no indication for overdispersion during model fitting. For both visibility and relative activity, we ran the models with the full random structure without model selection (Barr et al. 2013). To estimate the significance of potential predictors, we applied Type III Wald χ^2 tests (Herr 1986). All tests were two-tailed with alpha set to 0.05.

Data availability The datasets used in the current study are available from the author on reasonable request.

Results

We found that tadpole behavior changed considerably during early ontogeny, and the magnitude of this change was strongly affected by both the predation-cue treatment and food availability (Table 1; Figs. 1 and 2).

Visibility was significantly affected by the three-way interaction between food availability, chemical cues indicating predation risk and time (Table 1 and Fig. 1). When food availability was limited and predators were absent, the proportion of tadpoles hiding in the leaf litter remained similar throughout larval development, whereas it decreased somewhat in the ad libitum food treatment. Exposure to predation threat led to higher proportions of tadpoles hiding in the leaf litter as compared to tadpoles reared in the absence of predators, but only at later developmental stages and especially when food availability was limited.

The proportion of active tadpoles was overall higher when food was limited, especially so in young tadpoles, and

Table 1 Test statistics and significance of the investigated predictors from the fitted models. GLMMs were fitted with either binomial (in the case of visibility) or beta binomial (in the case of relative activity) error distribution

Response variable	Predictors	χ^2	df	P
Visibility	Predator	1.631	1	0.201
	Food	30.109	1	<0.001
	Time	74.231	1	<0.001
	Food \times time	48.140	1	<0.001
	Predator \times time	33.461	1	<0.001
	Predator \times food	12.859	1	<0.001
	Predator \times food \times time	9.527	1	0.002
Relative activity	Predator	60.552	1	<0.001
	Food	64.911	1	<0.001
	Time	40.911	1	<0.001
	Visibility	12.945	1	<0.001
	Food \times time	7.014	1	0.011
	Predator \times time	0.478	1	0.496
	Predator \times food	0.807	1	0.428
	Predator \times food \times time	0.012	1	0.887

decreased during the course of larval development (Table 1 and Fig. 2). However, the interaction between time and food availability was also significant: tadpole activity decreased over larval development more steeply when food was limited than in the ad libitum treatment. Finally, tadpoles exposed to cues indicating predation risk were less active at all times and in both food treatments than their conspecifics raised in the absence of predators.

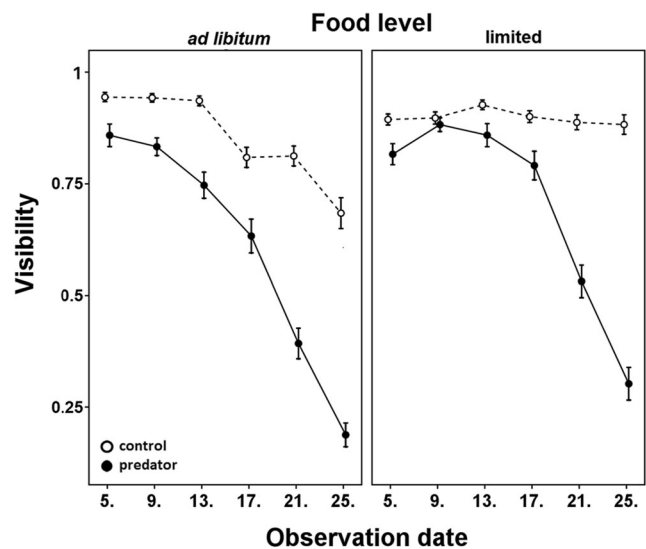
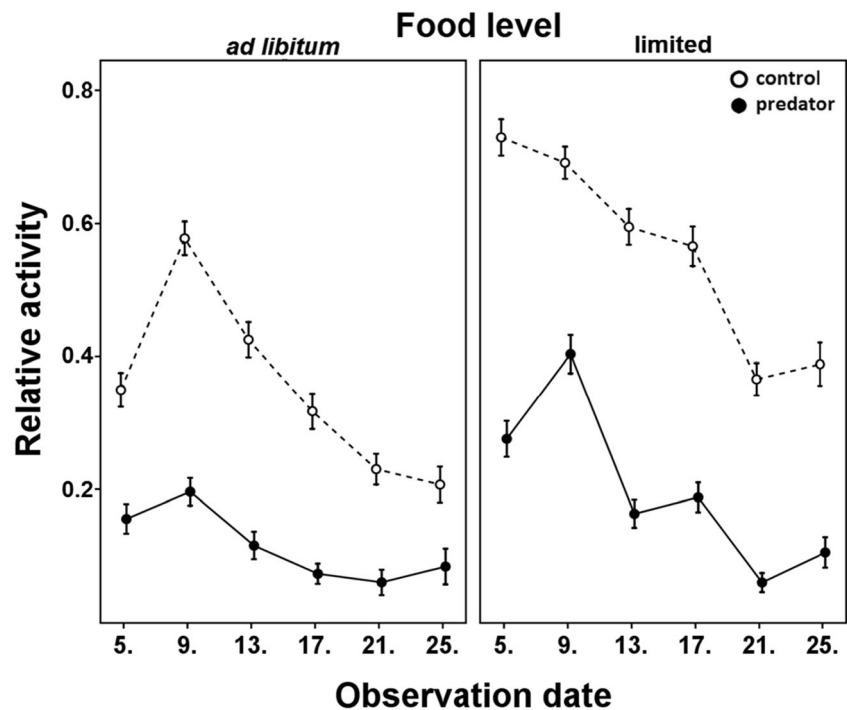


Fig. 1 Proportion of occasions when tadpoles were visible (mean \pm SE). Observation date indicates the days after start of the experiment (i.e., 2 days after hatchlings reached the free-swimming state). Solid lines and filled symbols indicate treatment groups where cues on predation threat were present, whereas dotted lines and open symbols represent treatment groups where cues on predation threat were absent

Fig. 2 Proportion of occasions when tadpoles were active (mean \pm SE). Observation date indicates the days after start of the experiment (i.e., 2 days after hatchlings reached the free-swimming state). Solid lines and filled symbols indicate predator-cue treatment groups, while dotted lines and open symbols represent treatments groups where cues on predation threat were absent



Discussion

In this study, we found that behavioral responses of common toads to variation in food availability and in predation risk change during larval development. Common toad tadpoles reacted to cues indicating predation risk by hiding more in the leaf litter, but this response was context-dependent. Hiding is likely to lower detectability and encounter rates with potential predators, thereby elevating tadpole survival probabilities (Lima and Dill 1990). However, when food was limited, tadpoles were more visible and active, indicating that individuals raised in poor conditions followed a more risk-taking strategy (e.g., Milinski and Heller 1978; Skelly 1995). These results align to findings of previous studies on bufonid tadpoles and larvae of other anurans (Laurila et al. 1997, 1998; Almeida et al. 2011; Nunes et al. 2014) and confirm that chemical cues indicating predation risk and food limitation together shape tadpoles' behavior. While previous studies rarely considered tadpole age when studying behavioral responses to environmental change, the significant three-way interaction between predator-cue treatment, food availability, and time documented here delivers unequivocal proof that integrated responses to predation risk and resource availability can change during tadpole ontogeny.

Tadpoles reduced both their visibility and activity during larval development and in response to predation risk, but the effect of age substantially affected their antipredator behavior. Although some studies reported similar trends in tadpole behavior through ontogeny (Bennett et al. 2013; Touchon et al. 2013), others documented a temporal increase or no changes

in the activity of tadpoles in response to the presence of predators (Eklöv and Werner 2000; Jara and Perotti 2010; Wilson and Krause 2012; Nunes et al. 2014). This variability in the observed behavioral responses may be the result of the different experimental setups used (Skelly and Kiesecker 2001; Winkler and Van Buskirk 2012) or of differences in antipredator strategies among prey species (Kats et al. 1988; Van Buskirk 2000; Relyea 2001; Relyea 2003; Relyea and Auld 2005). Newts, which we used as predators in our experiment, swallow their prey in one piece and may therefore become gape-limited, making them inefficient predators of large tadpoles (Wilbur et al. 1983; Urban 2007b, 2008). Nonetheless, we did not observe decreasing effect of this gape-limited predator either on tadpole activity or on visibility. Lowered responses to cues indicating predation risk in large tadpoles may have been lacking because we provided cues originating from the predator along with cues from injured conspecifics. Prey-borne cues alone can induce strong responses in tadpoles, especially if they are paired with the smell of a predator (Marquis et al. 2004; Hagman et al. 2009; Hettyey et al. 2015). Also, cues from injured conspecifics indicate danger, irrespective of the presence of a potentially harmless predator, hence the maintenance of intense behavioral antipredator responses.

Another potential explanation for the variability of previous findings may be related to different tadpole densities used in different experiments, which is known to affect tadpole behavior (including antipredator responses) substantially (Relyea 2004; McClure et al. 2009; Smith and Awan 2009; Preston and Forstner 2015). Toad tadpoles are gregarious, forming aggregations in natural ponds consisting of up to

several thousand individuals, and developing tadpoles can benefit from being in a group through dilution effects in the presence of predators (e.g., Watt et al. 1997). Following previous studies (Mirza et al. 2006; Smith et al. 2008; Jara and Perotti 2010), we observed individual tadpoles during the behavioral observations to maximize the number of statistical units and avoid the confounding effect of the presence of conspecific competitors on toxin production (Bókony et al. 2018), which could also have altered the advantage of exhibiting plastic behavioral responses to predation risk when tadpoles are kept in groups. We propose that while the observed ontogenetic change in the interactive effect of predation risk and food availability on antipredator behavior could indeed be further modified by the presence of conspecifics, our results provide well-founded experimental evidence for the significant impact of larval age on antipredator behavior when the confounding effect of competition or social learning between conspecifics is negligible.

We know of only a few studies in which age-dependent changes in behavioral trade-offs between foraging and predation risk avoidance were observed in amphibians during larval ontogeny. Laurila et al. (1998) found that *B. bufö* tadpole activity is affected by predator presence and day since feeding, and that these relationships change with time. They included time as a factor in their study; however, they did not address the question whether behavior changes with age, but rather how behavior changes between feeding occasions with time. Bennett et al. (2013) showed that *Lithobates pipiens* tadpoles displayed decreased activity with increasing predation risk, but no persistent response to different levels of food availability. Nonetheless, they reported time-dependent changes in the strength of behavioral antipredator responses and resource-dependent adjustments of behavior to food availability early on, but not late in larval development. Skelly and Werner (1990) investigated *Anaxyrus americanus* (formerly *Bufo americanus*) tadpoles, and they detected a temporal increase in predator avoidance (as we did), while food manipulation effects were non-significant. In these studies, the three-way interaction between food availability, predation risk, and age did not affect tadpoles' behavior or was not specifically investigated, so it may be possible that such patterns are rare and/or species specific. Similarly, previous findings in non-anuran organisms indicated the presence of ontogenetic changes in antipredator behavior (Dahlgren and Eggleston 2000; Dangles et al. 2007; Wohlfahrt et al. 2007), but the limited number of case studies does not allow for general conclusions about its importance yet.

Predation risk can have an influence on developmental rate, size at metamorphosis, the timing of metamorphosis, or the combinations of these traits (Higginson and Ruxton 2009; Kurali et al. 2016). A number of antipredator strategies are possible under food restriction, including accelerated development with no morphological or behavioral response, behavioral or morphological responses by prey, or a combination of

both types of responses coupled with slower development rate. For instance, it can be expected that defensive behavioral responses significantly decrease with increasing body size (e.g., Urban 2007b, for a non-anuran example see: Roth and Johnson 2004), resulting in an intertwined, multi-trait response to the presence of predation threat in suboptimal feeding regimes. However, even in this case, a causal relationship may persist between the predictive environmental cue and given traits, and changes in specific traits may not be adjusted to each other. In Kurali et al. (2016), we found that tadpoles reached metamorphosis earlier with lower body mass in the presence of simulated predation risk and limited food availability, in which case the reduced body size can be interpreted as a cost of accelerated development or lowered activity (Skelly and Werner 1990; Lardner 2000; Relyea and Auld 2005). However, in the absence of predator-cues, our current findings indicate that tadpoles maintained a higher activity even at the later stage of their larval development when food was scarce, and, thus, they were able to compensate for the lower energy intake by exhibiting increased foraging effort and delayed metamorphosis (Kurali et al. 2016).

In conclusion, we found that antipredator behavioral responses change during the ontogeny of *B. bufö* tadpoles and that these age-dependent changes in behavioral responses may also depend on resource availability. This work provides supporting evidence for the importance of both external and internal factors in the study of inducible defenses in amphibian model organisms and demonstrates that care should be taken when comparing behavioral antipredator responses between studies where ontogenetic stages and/or food availability were substantially different.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures involving animals in this study were approved by the Közép-Duna-Völgyi KTVF (KTVF 10350-2/2012) and the Ethical Commission of the MTA ATK NÖVI. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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