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Egg-laying environment modulates offspring responses to predation risk in an amphibian

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Abstract

Predator-induced plasticity has been in the focus of evolutionary ecological research in the last decades, but the consequences of temporal variation in the presence of cues predicting offspring environment have remained controversial. This is partly due to the fact that the role of early environmental effects has scarcely been scrutinized in this context while also controlling for potential maternal effects. In this study, we investigated how past environmental conditions, that is different combinations of risky or safe adult (prenatal) and oviposition (early post-natal) environments, affected offspring's plastic responses in hatching time and locomotor activity to predation risk during development in the smooth newt (*Lissotriton vulgaris*). We found that females did not adjust their reproductive investment to the perceived level of risk in the adult environment, and this prenatal environment had generally negligible effect on offspring phenotype. However, when predator cues were absent during oviposition, larvae raised in the presence of predator cues delayed their hatching and exhibited a decreased activity compared to control larvae developing without predator cues, which responses are advantageous when predators pose a threat to hatched larvae. In the presence of predator cues during oviposition, the difference in hatching time persisted, but the difference in general locomotor activity disappeared between risk-exposed and control larvae. Our findings provide clear experimental evidence that fine-scale temporal variation in a predictive cue during and after egg-laying interactively affects offspring phenotype, and highlight the importance of the early post-natal environment, which may exert a substantial influence on progeny's phenotype also under natural conditions.

Introduction

Environmental variability shapes organisms' traits by inducing plastic responses to predictive environmental cues and thereby greatly contributes to the evolution and maintenance of phenotypic variation in nature (West-Eberhard, 1989; Pfennig *et al.*, 2010; Laland *et al.*, 2014). Predation threat is one of the most consequential environmental effects that elicits plastic phenotypic responses (Lima & Dill, 1990; Tollrian & Harvell, 1999; Werner & Peacor, 2003), and in accordance with theoretical models of adaptive plasticity (Hoyle & Ezard, 2012; Ezard *et al.*, 2014; Leimar & McNamara, 2015), the expression of inducible defences is expected to be the highest when both trans- and within-generational environmental cues indicate high predation risk. In this case, the trans-generational cue influences an organism's phenotype only if it is sufficiently accurate, and the integration of different sources of cues can serve as a reliable predictor of the coming selective environment (Leimar & McNamara, 2015). Predation threat present in the maternal environment may lead to the maximal expression of inducible defences in offspring (Mousseau & Fox, 1998; Uller, 2008), and allows progeny to possess certain defences before its own adaptive responses develop (Jablonka & Lamb, 2005). Alternatively, trans-generational plasticity

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can also be favoured by natural selection when there is limited opportunity for within-generational plasticity and phenotype–environment mismatches incur high costs (English *et al.*, 2015). Previous studies provided ample experimental evidence for the presence of maternal effects that increase the quality or performance of offspring in risky environments (e.g. Agrawal *et al.*, 1999; Storm & Lima, 2010; Bestion *et al.*, 2014), even if this effect has been found to be generally weak (Uller *et al.*, 2013).

Marshall & Uller (2007) recently emphasized that not all temporal and spatial environmental scenarios favour the evolution of such anticipatory maternal responses, that is maternal effects are context dependent, depending on multiple spatial and temporal factors. In line with that, some studies reported the lack of trans-generational effects in some phenotypic traits (Beaty *et al.*, 2016), whereas others observed opposite within- and trans-generational effects (Walsh *et al.*, 2015) and even maladaptive consequences of maternal effects (Haussmann *et al.*, 2012; McGhee *et al.*, 2012). A potential mechanism by which both beneficial and detrimental maternal conditions can be transferred to offspring is related to maternally derived stress (MDS; Love *et al.*, 2013; Sheriff & Love, 2013) mediated by glucocorticoid hormones (GCs; Denver, 2009; Maher *et al.*, 2013; Kirschman *et al.*, 2017). Increased level of predation risk can increase maternal GC levels (Meylan & Clobert, 2005; Giesing *et al.*, 2011), and embryos are known to be sensitive to maternal GCs, leading to physiological, morphological and/or behavioural changes in their phenotype during development (Sheriff *et al.*, 2010). Besides their direct effects, maternal GCs may also alter the quality/quantity of resources invested by the females into their progeny (Sinervo & DeNardo, 1996). MDS-induced responses can be adaptive for the offspring if the environment is predictable and selection has opted the MDS to modify offspring phenotype in a way that is best suited to that environment, or MDS can induce reduction in offspring quality and therefore be costly for the offspring (but potentially beneficial for the mother; Sheriff & Love, 2013).

Not only maternal effects, but early exposure to predictive environmental cues may also induce long-lasting phenotypic changes in offspring. Recent evidence suggests that the effect of past and current within-generational environments is linked (Beaman *et al.*, 2016) and responses induced in different phases during development may shape many phenotypic features together (Dingemanse & Wolf, 2013). Fawcett & Frankenhuis (2015) also proposed an information-based conceptual framework for understanding the conditions under which the evolution of sensitive windows in development can be expected, that is periods or stages during ontogeny which have a particularly strong influence on individuals' phenotype compared to other periods or stages. The authors suggested that organisms are more likely to respond with phenotypic changes to environmental cues early in life and natural selection should favour a decline in plasticity across lifetime if predictive cues are frequent and informative, individuals are relatively unconstrained in expressing phenotypic adjustment in early ontogenic stages, and environment–phenotype mismatches are costly (Fawcett & Frankenhuis, 2015). Also, the more reliably environmental cues predict the future environment, the faster plasticity may decline during ontogeny (Frankenhuis & Panchanathan, 2011). There are numerous studies which investigated how developmental conditions influenced plasticity in juveniles and adults in the context of adaptive inducible defences (Vonesh & Warkentin, 2006; Touchon *et al.*, 2013, 2015). However, experimental studies are still scarce about how exposure to predator cues in the prenatal and early post-natal environments may contribute to the expression of predator-induced phenotypic responses, even though the importance of temporal variation in the presence of predation risk has been proposed previously (e.g. Orizaola & Braña, 2005; Hoverman & Relyea, 2007; Lehman & Campbell, 2007).

In this study, we examined how chemical cues of predation threat – present in the maternal and/or oviposition environments – interactively affected reproductive investment of adults (number and size of deposited eggs) and plastic responses of larvae to predation threat in hatching time and locomotor activity in the smooth newt (*Lissotriton vulgaris*), one of the most widespread caudate species in Europe (Arntzen *et al.*, 2009). In smooth newts, adults share the same aquatic environment with their offspring during the reproductive period, but, especially at the start of the breeding season, females may visit several ponds which are in close spatial proximity (Weddeling *et al.*, 2004), but vary largely in their predator fauna (Tóth, 2015; Bókony *et al.*, 2016). Also, several predators of newt eggs and larvae readily move among ponds (e.g. imagos of water beetles and backswimmers) or emerge from overwintering after smooth newts have started their reproductive period (e.g. larval dragonflies). Because of that, the maternal environment may not predict larval conditions accurately; thus, our initial prediction was that maternal environment will have generally little effect on offspring phenotype. We also predicted that the oviposition environment, in which offspring encounter predator cues directly and which is likely to reflect future predation threat more accurately, will substantially affect the developmental trajectory of larvae, manifesting in adaptive responses such as delayed hatching (in accordance with life history theory; Werner, 1986; see also in Ireland *et al.*, 2007; Wojdak *et al.*, 2014) and decreased locomotor activity (in accordance with the generally adaptive value of reduced activity under predation risk; Lima, 1998; see also in Van Buskirk & Schmidt, 2000; Schmidt & Van Buskirk, 2005). Finally, we also expected that the presence of predator

cues in the subsequent embryonic and larval environment would further enhance previously induced antipredator responses of the offspring. For the case when cues in the oviposition and developmental environments were mismatched, we predicted that larval phenotype will be more affected by the presence of predator cues in the oviposition environment than in the subsequent developmental environment (following Fawcett & Frankenhuis's (2015) idea), but, due to the interrupted or shortened exposure, the expressed levels of responses will be intermediate.

Materials and methods

Animal collection and housing

Adult smooth newts (63 males and 63 females) were captured using underwater traps and dip-netting from three ponds in the north-eastern part of the Pilis Mountains, Hungary, on 13 April 2015 (Table S1) and transported to the laboratory. We randomly assigned males and females originating from the same pond into pairs and housed each pair in a transparent plastic container (30 (L) × 20 (W) × 16 (H) cm), filled with approx. 4 litres of aerated reconstituted soft water (RSW) (American Public Health Association, 1985). In the containers, we provided one thread of *Elodea* for egg-laying and a plastic pot reaching out of the water as a shelter and a climbing surface. Animals were kept under a 13(L): 11(D) photoperiod at 17.94 ± 0.63 °C (mean ± SD) ambient temperature and fed *ad libitum* with live *Tubifex* worms throughout their captivity. After the adult treatment ended (see below), we nonlethally anesthetized the animals by placing them into a 0.1% solution of MS-222 (CAS: 886-86-2; Sigma-Aldrich Co., St. Louis, MO, USA), then took photographs of the adults using a Canon Powershot SX50 HS digital camera (Canon Inc., Tokyo, Japan) and measured their body mass to the nearest mg using a digital scale (Ohaus Pioneer PA213; Ohaus Corp., Parsippany, NJ, USA). After full recovery, adult newts were transported back to their site of capture. During the experiment (see below), we collected eggs together with *Elodea* leaves on which they were deposited. After unwrapping leaves from the eggs (if necessary), we photographed and subsequently kept the eggs at 18.18 ± 0.47 °C ambient temperature in small plastic boxes (13.1 × 8.7 × 4.0 cm) filled with approx. 0.1 L of RSW (with or without predator cues; see below). We checked eggs each day until all larvae hatched and removed nondeveloping or mouldy eggs. After hatching, we kept larvae under identical conditions as the eggs, except that we provided zooplankton collected from a small pool at the experimental station to feed the hatched larvae *ad libitum*. After video-recording (see below), all animals were released at their site of capture.

Invertebrate predators were collected using underwater traps and dip-netting between 9 March and 13 April 2015, from six ponds in Hungary (the number of collected individuals and coordinates of the ponds are shown in Table S1). We captured adult *Acilius sulcatus* (Coleoptera: Dytiscidae) water beetles (body mass [mean ± SD] measured at the end of the experiment: 320.14 ± 42.42 mg, $N = 7$), adult *Graphoderus* sp. (Coleoptera: Dytiscidae) water beetles (body mass: 225 ± 23.29 mg, $N = 5$) and southern hawker, *Aeshna cyanea* (Odonata: Aeshnidae) larvae (instars F1–F3; body mass: 965.17 ± 103.8 mg, $N = 12$), and used them for producing predator chemical cues during the experiment. These invertebrates are important predators of caudates' eggs and larvae (Miaud, 1993; Tóth, 2015), and aeshnid larvae, especially in the final instar stage, pose a serious threat to adults as well. We kept three or four water beetles in plastic boxes (21 × 15 × 12 cm) filled with 1.5 or 2 L of RSW, respectively, together with three or four pieces of plastic mosquito net as a climbing surface. This '0.5 litre per individual' ratio was maintained throughout the study (4 of 16 beetles died in different boxes for unknown reasons before the video trial). Dragonfly larvae were housed individually in 0.5-L plastic cups filled with approx. 0.2 L of RSW, and plastic sticks were provided as perching sites. Some dragonfly larvae reached the F0 instar stage and underwent metamorphosis during the course of the study; these larvae were then replaced in order to have 12 dragonfly larvae for predator-cue collection. Predators were kept under a 13(L): 11(D) photoperiod at 18.07 ± 1.66 °C ambient temperature and were fed *ad libitum* with *Tubifex* worms throughout the study. Their housing water, when not used in the experiment to provide predator cues, was changed at least once a week.

All sampling procedures and experimental manipulations reported in this study were reviewed and specifically approved by the national authority of the Middle-Danube-Valley Inspectorate for Environmental Protection, Nature Conservation and Water Management, Hungary, who issued the permission to capture and conduct experiment on the animals (KTF: 2771-3/2015). Furthermore, all applicable institutional and national guidelines for the care and use of animals were followed during the study.

Experimental procedures

We applied a fully crossed three-way factorial design to disentangle the potential effects of chemical cues of predation risk in the maternal, oviposition and developmental (i.e. embryonic/larval) environments (Fig. 1). On Day 1, we placed fresh *Elodea* threads for egg-laying into the containers of those pairs which started depositing eggs. On Day 2, we placed new *Elodea* threads into their containers and randomly allocated half of the pairs to the 'predator-cue' treatment group, whereas the other half to the 'no predator-cue'

treatment group ('adult environment' treatment). In the former, we added 80 mL of a mixture of predator cues daily to the housing water, whereas in the latter the same amount of RSW was administered. The predator-cue mixture was prepared by pooling the housing water of the water beetles and dragonfly larvae; thus, it contained both digestion- and continuously released predator-borne cues (*sensu* Hettyey *et al.*, 2015). This treatment lasted for 5 days, during which females could get accustomed to their environment and adjust their reproductive investment (in the form of depositing different numbers of eggs and/or provisioning different amounts of GCs/nutrients into the eggs) to the presence or absence of predator cues. Although both parents received the same predator cues during this period, we regarded any effect of this adult environment as maternal due to the lack of evidence for sperm-mediated effects in antipredator responses in newt larvae (note also that females could already have mated prior to collection, and multiple paternities are common in this species; Jehle *et al.*, 2007). All eggs laid during the adult environment treatment were discarded. On Day 7, a new *Elodea* thread was placed into the containers, and half of the pairs in each treatment group were randomly allocated to either the 'predator-cue' or the 'no predator-cue' treatment groups ('oviposition environment' treatment). This part of the experiment lasted for 24 h, after which we collected, counted and photographed the deposited eggs. In total, 52 females laid eggs continuously during these two treatments. In the above scenario, the adult environment treatment corresponds to the manipulation of the prenatal environment, whereas the oviposition treatment to the manipulation of both the prenatal and early post-natal environment; this latter treatment could inevitably induce both final maternal or first offspring responses to cues in the shared environment. After counting and taking photographs of the laid eggs ($n = 429$), half of the eggs of each female were haphazardly allocated to either the 'predator-cue' or the 'no predator-cue' treatment groups ('egg and larval environment' treatment). In the 'predator-cue' treatment group, eggs and larvae received 2 mL of the mixture of predator cues, whereas in the 'no predator-cue' treatment group an equivalent amount of RSW was added to the rearing boxes every day. In this scenario, the egg and larval environment treatment corresponded to the manipulation of the post-natal (developmental) environment of the offspring. Eggs of each female in each treatment group were kept together ('cohort'), and hatching events in the cohorts were checked and recorded every day (Table S2). We fed larvae ($n = 413$) with zooplankton *ad libitum* until the video trial, which took place between 13 and 15 May (age of the larvae ranged between 3 and 12 days). Offspring survival was similarly high in all treatment combinations and had an overall value of 95.32%. Spatial randomization of container position has been applied throughout the experiment.

Video analysis and data processing

We recorded larval movement using two Sony DSC-HX200V (Sony Inc, Tokyo, Japan) cameras set to 1920 × 1080p resolution and 50 frames per second. Cameras were fixed approx. 50 cm above a horizontal platform, onto which we placed nine experimental arenas. These arenas were 13.1 × 8.7 cm in size, filled with 0.1 L RSW to which we added 2 mL of the mixture of predator cues or RSW, in accordance with the developmental environment of the randomly selected larvae. This means that the test environment and the developmental environment were identical for each larva in terms of the presence or absence of predator cues. The prepared experimental arenas were placed randomly under the cameras' view, then the larvae were put individually into their assigned arenas. The set-up was identical to the housing conditions of larvae during development, so we later discarded the first three minutes from the footages as a short period of acclimatization, during which the allocated larvae could get accustomed to their surroundings undisturbed by the observer. The next 25 min were analysed using the software Ctrax (Branson *et al.*, 2009), resulting in the analysis of 75 000 frames for each individual. We excluded those videos from later analyses in which the software's algorithm could not track the focal individual in more than 50 frames, and we also excluded those females (and their progeny) which did not have at least one offspring in each 'developmental environment' treatment group due to the former criterion. This resulted in a sample size of 349 larvae of 46 females in the analysis of locomotor activity. The obtained raw coordinates (measured with ± 0.1 mm precision) were smoothed to eliminate any tracking noise using the path smoother algorithm of the 'RSEE' R package (Hen *et al.*, 2004). We calculated total duration of moving, total distance covered, mean duration of bursts (i.e. short periods of rapid movements), mean distance covered during bursts, maximum duration of bursts, maximum distance covered during bursts and maximum velocity (in mm/frame; Table S2). Because none of the measured traits were *a priori* expected to be of special relevance (for instance, aeshnid predators do not induce higher maximum velocity in amphibian larvae; Van Buskirk & McCollum, 2000; Gvoždík & Smolinský, 2015; Johnson *et al.*, 2015), we used a principal component analysis (PCA) on all seven positively correlated variables (pairwise correlations [r_s] ranged from 0.37 to 0.91) to calculate overall locomotor activity of the larvae. Prior to PCA, the first six measures, characterized by right-skewed distributions, were log-transformed to reduce the influence of extreme values and to ensure

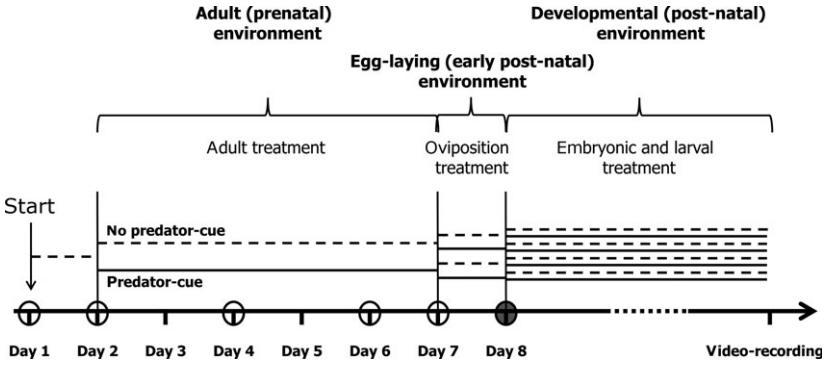

Fig. 1 Schematic diagram of the experiment. Adult treatment consisted of a daily addition of 80 mL of RSW ('no predator-cue') or an equivalent amount of housing water of predators ('predator-cue') to reproducing adults' housing water for five days, while the similar oviposition treatment lasted only for a single day. Afterwards, only those eggs that were deposited overnight by each female were collected and divided into two cohorts; these eggs then received either the 'predator-cue' or the 'no predator-cue' treatment during the egg and larval treatment until the video-recording. Empty circles indicate days when new *Elodea* threads were supplied to the pairs, the circle filled with grey denote the day when deposited eggs were collected. Larvae hatched between Day 20 and Day 27, while the video-recording took place from Day 30 to Day 32.

that variables are linearly, or at least monotonically, related to one another (Quinn & Keough, 2002). The first principal component ('locomotor activity PC1') represented 69.59% of the variation with all parameters loading positively on this component (Table S3), so that higher PC1 scores represented individuals exhibiting higher overall locomotor activity. The second principal component ('locomotor activity PC2') explained an additional 13.17% of the variation. Time of moving and total distance covered loaded positively, whereas all other original variables loaded negatively on PC2 (Table S3). Hence, higher PC2 scores represented individuals which moved for a longer period and covered a longer distance during the trial, but had shorter movement bursts, covered smaller distances during bursts and had lower maximum speed.

We measured snout-to-vent length (SVL) of each female from digital images using ImageJ 1.48v (US National Institutes of Health, Bethesda, MD, USA; Abramoff *et al.*, 2004). Similarly, length along the long axis of symmetry and the largest width were measured on the photographed eggs (without the jelly coat; Table S2). We performed PCA on the collected eggs' length and width and considered the first principal component as a proxy for egg size in the subsequent analysis. Both dimensions of size loaded positively on the first component (both loadings equalled 0.71); that is higher values indicated longer and wider eggs, and this component (egg size, henceforward) accounted for 79.01% of the total variance.

Statistical analysis

We conducted all statistical analyses in R 3.3.2 (R Core Team 2016). We fitted a linear mixed-effect (LME) model on the number of eggs deposited at the end of the oviposition treatment using the 'nlme' R package (Pinheiro *et al.*, 2016), in which unequal variances in the dependent variable between ponds could be accounted for by adding 'weights' with 'varIdent' to the model. We included female SVL (centred to the mean), 'adult environment', 'oviposition environment' and the interaction of the latter two as explanatory variables, whereas 'pond' was added as a random variable to the model. We fitted LME models using the 'lme4' R package (Bates *et al.*, 2015) to investigate how female SVL (centred to the mean), 'adult environment', 'oviposition environment' and the interaction of the two treatments affected egg size. Into these models, we also included 'female identity' nested into 'pond' as a random term. We fitted a mixed-effect Cox proportional hazard model on individual hatching time using the 'coxme' R package (Therneau, 2015). Eggs which did not hatch due to mortality were also included into the dataset as right-censored data ($n = 13$). Into this hazard model, we included female SVL (centred to the mean), 'adult environment', 'oviposition environment', 'developmental environment' and all possible interactions between these treatments (including the three-way interaction) as potential explanatory variables. We added 'female identity' nested into 'pond' as a random term to this model. We used LME models to examine how larval locomotor activity PC1 and PC2 scores were affected by female SVL (centred to the mean), maximum age in the cohort (centred to the median), 'adult environment', 'oviposition environment', 'developmental environment' and all possible interactions between these treatments (including the three-way interaction). As we could not measure larval body length with sufficient accuracy from video recordings (please note that the camera was fixed 50 cm above the arenas), we used the maximum age in the cohort as a proxy to

account for some of the variation between cohorts originating from differences in hatching time. Maximum age reflected the age of the oldest larva in a given cohort at the time of the video trial (in days); by including this explanatory variable into the models, we aimed to test for differences in antipredatory behaviour between treatments over and above the effects of the differences in larval age. In these models, 'female identity' nested into 'pond', 'trial', 'position of the container during trial' and 'camera' were also added as crossed random factors. Compliance with the proportional hazard assumption was confirmed in the fitted hazard model, whereas requirements of the fitted LME models were checked by plot diagnosis. Random variables were excluded from the random term if they had negligible effect on model fit based on likelihood ratio tests (Pinheiro & Bates, 2000); when all random effects were found to be negligible, LME models shrank to linear models (only in the case of number of eggs laid). To estimate the significance of potential predictors in the fitted (full) models, we applied type II Wald χ^2 tests. *Post hoc* comparisons with Tukey adjustment were performed on all possible pairs of the significant predictor(s) using the 'lsmeans' R package (Lenth, 2016). All tests were two-tailed with alpha set to 0.05. Data used in the statistical analyses are available from Figshare (https://figshare.com/s/16f1bab892aa5dd5f7fa).

Results

Number of eggs laid at the end of the oviposition treatment was not affected by adult environment, oviposition environment or their interaction, or by female SVL (all $P \geq 0.101$; Table 1). Egg size was positively related to female SVL ($\chi^2_1 = 10.33$, $P = 0.001$; Table 1), indicating that larger females deposited longer and wider eggs (β with 95% CI: 0.15 [0.06–0.23]). Adult and oviposition environments (either by themselves or in interaction with each other) had no significant effect on egg size (all $P \geq 0.364$).

Hatching time was significantly affected by the interaction of oviposition environment and developmental environment ($\chi^2_1 = 7.64$, $P = 0.006$; Table 1). Embryos which were raised in the presence of predator cues were significantly more likely to hatch later than those raised in the absence of predator cues, but this effect was less pronounced in the presence than in the absence of predator cues in the oviposition environment (estimated differences ± SE: 0.38 ± 0.14 days, $z = -2.62$, $P = 0.043$ and 1.22 ± 0.24 days, $z = -5.0$, $P < 0.001$, respectively; Fig. 2). These differences corresponded to 1.51 times the rate of hatching in the control than in the presence of predator cues when the oviposition environment was risky (Hazard Ratio with 95% CI: 1.51 [1.14–2.01]), and 2.85 times the rate of hatching in the control than in the presence of predator cues when the oviposition environment was safe (Hazard Ratio with 95% CI: 2.85 [2.09–3.90]). Not surprisingly, embryos which experienced predation risk in both environments also hatched later than those which did not encounter predator cues in either environments (−1.14 ± 0.31 days, $z = -3.66$, $P = 0.002$); all other comparisons were found to be nonsignificant (all $P > 0.087$). Female SVL and adult environment (either by itself or in interaction with other environments, including the three-way interaction between treatments) had no significant effect on hatching time (all $P \geq 0.137$).

Locomotor activity PC1 was influenced by the interaction of oviposition and developmental environments ($\chi^2_1 = 9.34$, $P = 0.002$; Table 1). When predator cues were present in the oviposition environment, larval activity was unaffected by the developmental environment, but when predator cues were absent in the oviposition environment, larvae raised in the presence of predator cues exhibited significantly lower activity compared to those raised in the control (estimated difference ± SE: −1.0 ± 0.35, $t = -2.83$, $P = 0.025$; Fig. 3). All other comparisons were found to be nonsignificant (all $P \geq 0.156$). Female SVL and maximum age in the cohort were negatively related to larval PC1 scores (SVL: $\chi^2_1 = 4.34$, $P = 0.037$; maximum age: $\chi^2_1 = 4.55$, $P = 0.033$), indicating that larger females had generally less active offspring (β with 95% CI: −0.11 [−0.21 to −0.01]) and larvae from cohorts in which maximum age of individuals was higher had a lower locomotor activity (−0.28 [−0.54 to −0.02]). The adult environment had no effect on PC1 scores, either by itself or in interaction with other predictors, and the three-way interaction between treatments had no significant effect either (all $P \geq 0.407$). Locomotor activity PC2 was significantly affected by the developmental environment ($\chi^2_1 = 7.62$, $P = 0.006$; Table 1): larvae raised in the absence of predator cues had higher scores than those raised in the presence of such cues (β with 95% CI: 0.30 [0.11–0.49]). Female SVL, maximum age in the cohort, or adult and oviposition environments (either by themselves or in interaction with each other or with developmental environment) had no significant effect on this variable, similarly to the three-way interaction between all treatments (all $P \geq 0.154$).

Discussion

Our results demonstrate that plastic responses of developing newt larvae to predation threat are substantially influenced by oviposition conditions, but not by the maternal environment. When eggs were laid in a predator-free environment, offspring showed plastic responses to predation risk in the subsequent developmental environment as predicted by previous works, that is by delaying their hatching (Ireland *et al.*, 2007; Wojdak *et al.*, 2014) and decreasing their locomotor activity (Van Buskirk & Schmidt, 2000; Schmidt & Van

Table 1 Test statistics and significance of the investigated explanatory variables from the fitted models.

Response variable	Random term	Explanatory variables	χ^2	d.f.	*P*
Number of eggs laid	–	SVL	2.70	1	0.101
		Adult environment	0.03	1	0.869
		Oviposition environment	1.17	1	0.279
		Adult env. × Oviposition env.	0.05	1	0.823
Egg size	Female identity: 0.86 [0.65–1.02]	**SVL**	**10.33**	**1**	**0.001**
		Adult environment	0.82	1	0.364
		Oviposition environment	0.03	1	0.872
		Adult env. × Oviposition env.	0.12	1	0.724
Hatching time	Female identity: 0.52 [0.37–0.72]	SVL	0.11	1	0.744
		Adult environment	0.03	1	0.857
		Oviposition environment	1.31	1	0.252
		Developmental environment	**42.47**	**1**	**<0.001**
		Adult env. × Oviposition env.	1.17	1	0.279
		Adult env. × Developmental env.	0.21	1	0.650
		Oviposition env. × Developmental env.	**7.64**	**1**	**0.006**
		Adult env. × Oviposition env. × Developmental env.	2.21	1	0.137
Locomotor activity PC1	Female identity: 0.57 [0–0.83]	**SVL**	**4.34**	**1**	**0.037**
	Trial: 0.62 [0.27–0.99]	**Maximum age in the cohort**	**4.55**	**1**	**0.033**
		Adult environment	0.04	1	0.844
		Oviposition environment	0.19	1	0.660
		Developmental environment	0.63	1	0.426
		Adult env. × Oviposition env.	0.02	1	0.901
		Adult env. × Developmental env.	0.69	1	0.407
		Oviposition env. × Developmental env.	**9.34**	**1**	**0.002**
		Adult env. × Oviposition env. × Developmental env.	0.11	1	0.738
Locomotor activity PC2	Female identity: 0.22 [0–0.35]	SVL	0.14	1	0.704
	Camera: 0.30 [0.08–0.90]	Maximum age in the cohort	0.62	1	0.431
		Adult environment	0.02	1	0.889
		Oviposition environment	0.49	1	0.484
		Developmental environment	**7.62**	**1**	**0.006**
		Adult env. × Oviposition env.	0.07	1	0.791
		Developmental env. × Adult env.	0.02	1	0.890
		Developmental env. × Oviposition env.	2.03	1	0.154
		Adult env. × Oviposition env. × Developmental env.	0.14	1	0.704

Significant predictors are shown in bold; random effects are given in SD ± 95% confidence interval.

Buskirk, 2005). In the presence of predator cues during oviposition, offspring raised in risky environment also hatched later than the control larvae, but the difference in general activity completely diminished and larvae were similarly active irrespective of their developmental environment. Thus, predator cues in the egg-laying environment in our study did not enhance larval antipredator responses, but, contrary to our expectations, dampened the degree of behavioural response to predation threat in newt larvae.

Previous studies showed that many amphibians are capable of exhibiting innate antipredator responses (Wells, 2007), but in most studies females laid their eggs in an untreated (i.e. risk-free) environment, and thus provided little information about how the developmental window in this early post-natal phase is associated with the formation of specific phenotypic responses (Hoverman & Relyea, 2007). Lehman & Campbell (2007) found that caudate embryos responded to chemical cues of predatory caddisfly larvae by hatching earlier during their study, but such responses were induced only when embryos were exposed to predator cues from the first day of their development. Indeed, variation in conditions during critical stages or periods during development has been proposed by previous works to have long-lasting phenotypic consequences in many organisms' life, even if the exposure is of short duration and/or of weak intensity (see examples in Taborsky, 2006; Massot & Aragón, 2013; Mueller *et al.*, 2015; Ferrari *et al.*, 2015). Moreover, the presence of such sensitive windows early on during development accords well with relevant theory (Fawcett & Frankenhuis, 2015).

Nettle & Bateson (2015) recently emphasized the importance of distinguishing between two frameworks, within which adaptive developmental plasticity can be interpreted and explained. The proposed distinction is based on whether organisms are responding only to the

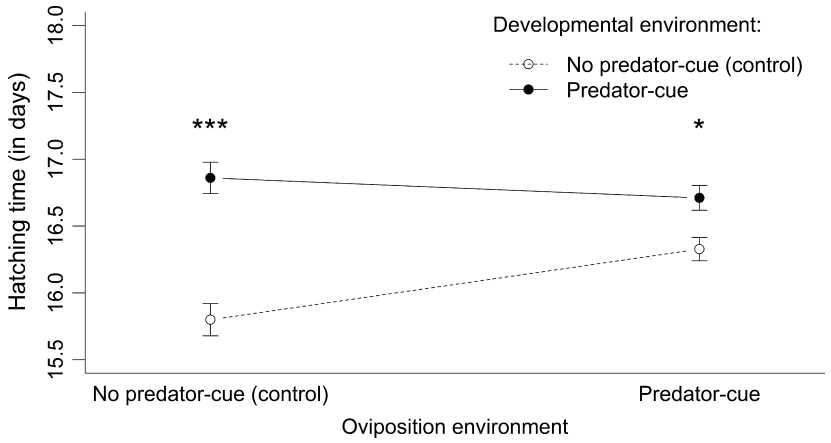

Fig. 2 Hatching time (mean ± SE) in both oviposition environment and both developmental environment treatment groups. Empty circles represent eggs that developed in the absence of predator cues during the developmental environment treatment, whereas filled circles denote eggs that were kept in the presence of such cues. Please note that the figure was drawn using the raw hatching data, while parameter estimates were obtained from a mixed-effect Cox proportional hazard model. The *post hoc* test comparing all possible contrasts was performed with Tukey adjustment; significant differences between risk-exposed and control eggs in the two oviposition environments are indicated by asterisks.

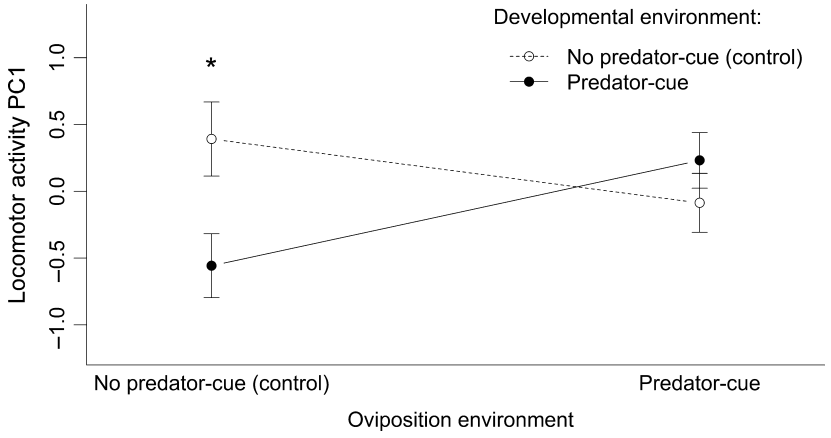

Fig. 3 Locomotor activity PC1 (mean ± SE) in both oviposition environment and developmental environment treatment groups. Empty circles represent larvae that developed in the absence of predator cues during the developmental environment treatment, whereas filled circles denote offspring that were raised in the presence of such cues. The *post hoc* test comparing all possible contrasts was performed with Tukey adjustment; the significant difference between risk-exposed and control larvae within the control oviposition environment is indicated by an asterisk.

information content of a particular cue that is present in their environment (informational interpretation), or individuals exhibit a direct physiological response to the environmental cue, which then permanently influence some aspects of their somatic state (somatic state-based interpretation). In principle, our results could be interpreted in either of these frameworks given that predator cues provided information about risk in the environment, but could also directly elicit a stress response in the tested individuals. Within the informational framework, we can assume that subsequently mismatched environments reduce the information content of given cues and that cues with higher information content elicit higher levels of response; thus, the largest phenotypic difference could be expected between larvae that were consistently experiencing control conditions and larvae that were consistently experiencing risky conditions (as we also assumed in our initial predictions). However, we did not find any indication for such a pattern in either hatching time or locomotor activity. Therefore, instead of focusing on the information content of the administered predator cues, we suggest that the somatic state-based framework provides a more plausible explanation for the observed phenotypic changes.

A possible mechanistic explanation for our findings could be related to a bimodal physiological stress response to predation risk (Maher *et al.*, 2013). When amphibian larvae are exposed to predators, individuals' neuroendocrine stress axis becomes suppressed, resulting in reduced locomotor and foraging activity (Crespi & Denver, 2004, 2005; Fraker, 2008; Fraker *et al.*, 2009). Maher *et al.* (2013) showed that in the case of short-term exposure to exogenous stress hormones, tadpoles adjusted their behaviour and enhanced their survival probability by reducing their exposure to predators. However, when tadpoles were treated over a longer period, adaptive changes were induced in morphology rather than in behaviour, and consequently facilitating their survival through a more efficient antipredator defence. In our study, such bimodal response may provide a plausible explanation why those newt larvae which encountered predator cues only after oviposition had lower activity than their control counterparts, but not those which were exposed to predator cues throughout their development (although the difference in exposure duration was only 24 h). Alternatively, from a hormetic framework perspective, first exposure to a mild stressor mitigated the level of stress response later in life (Costantini *et al.*, 2010), so that exposure to chemical cues of predators during egg-laying could buffer against exaggerated and, thus, maladaptive larval responses. A third possibility is that newt embryos learned to recognize the administered cues as those of a nonpredator organism during early post-natal exposure, since there was no risk reinforcement involved in our predator-cue treatment (i.e. the added mixture did not contain conspecific alarm cues). This phenomenon is termed 'latent inhibition' and has previously been demonstrated in anuran embryos (Ferrari & Chivers, 2009). However, this idea does not explain the consistent difference in hatching time between risk-exposed and control larvae in our study. Irrespective of the mechanism behind, our findings indicate that egg-laying can be a key ontogenic stage during which the expression of predator-induced responses can be altered in a potentially advantageous way (if any of the above explanations are valid). We propose that further studies should explicitly investigate the adaptive value of environmental effects during egg-laying by examining its consequences on growth rate and survival in various oviparous organisms. For instance, a limited developmental window of responsiveness in prey may correspond to the phenology of predators in natural habitats and can be viewed as an adaptive trait in response to predictable temporal variation in predation risk (see also in Lehman & Campbell, 2007; Fawcett & Frankenhuis, 2015).

Females and their offspring share the same environment during egg-laying, so that the effect of oviposition environment on larval phenotype can, in principle, be attributed to induced responses in both generations. Embryos in freshly laid eggs have been found to react to chemical cues in previous studies (e.g. Lehman & Campbell, 2007; Ferrari & Chivers, 2009), while maternal effects are known to be mediated often through egg size (e.g. Giesing *et al.*, 2011; Segers & Taborsky, 2012). Oviposition environment did not affect egg size in our study, indicating the absence of influential maternal effects in the observed predator-induced responses, but we lack information about whether stress hormones are *de novo*-synthesized in amphibian embryos or stress responses during early development can only be attributed to maternally deposited GCs (as in teleost fish; Nesan & Vijayan, 2013). Because of that, we cannot exclusively interpret these phenotypic changes as antipredator responses of the larvae; maternal responses to the egg-laying environment may also have contributed to these changes. However, in accordance with our prediction, we found no indication that the maternal environment would have affected either females' investment or offspring phenotype, not even when adults encountered predation risk prior to egg-laying. Although we showed that maternal condition affected egg size and larval activity in a way that larger females laid larger eggs and had less active larvae, the adaptive value of such maternal effect on offspring phenotype has recently been debated; some authors has argued that these positive relationships are rather physiological side effects (Marshall & Uller, 2007), whereas others have proposed that such condition-transfer effects can also reflect evolved parental investment strategies (Bonduriansky & Crean, 2017).

In this study, we found experimental evidence for the interactive effect of oviposition environment and developmental environment on offspring phenotype in a widespread amphibian species. We observed the strongest larval antipredator responses when predator cues had been absent in the adult and egg-laying environments; that is, adults were kept in control conditions and females deposited their eggs in a risk-free environment. However, our findings also showed that temporal variation in predation risk during reproduction leads to detectable shifts in offspring phenotype and highlight the potential importance of the early post-natal environment, which may exert a substantial influence on progeny's phenotype also under natural conditions in various species.

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Conflict of interest

The authors have no conflict of interest to declare.

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Supporting information

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

Table S1 Coordinates of the ponds and total number of animals collected for the experiment.

Table S2 Descriptives of individual characteristics measured during the study and used in the statistical analyses.

Table S3 Loadings of the calculated activity-related measures on locomotor activity PC1 and PC2.

Table S4 Parameter estimates and corresponding SEs in final models.

Data deposited at Dryad: https://doi.org/10.5061/dryad.432vj5j

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