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Adaptive plasticity in stressful environments: acidity constrains inducible defences in *Rana arvalis*

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ABSTRACT

Questions: How do environmental stressors affect the expression of adaptive phenotypic plasticity? Is there inter-population variation in these effects?

Hypothesis: Acid stress constrains the expression of inducible defences by decreasing investment in defences or by increasing the costs of investment. Organisms originating from neutral environments suffer more from acid stress than organisms originating from acid environments.

Organism: Tadpoles of *Rana arvalis*, originating from two different populations (acid and neutral). This species displays inducible defences in response to insect predators (here dragonfly larvae).

Methods: A laboratory experiment with a factorial design crossing two factors: predator presence (present vs. absent) and acidity (neutral vs. acid). We tested the effects of experimental treatment on tadpole morphology as well as age and size at metamorphosis.

Results: Tadpoles from the neutral origin population invested less in inducible defences (tail fin depth) in the acid than in the neutral treatment. In contrast, tadpoles from the acid origin population were able to respond equally well to predators in both pH treatments. pH-related costs differed between populations: while tadpoles from the neutral origin population suffered from acid stress in terms of reduced developmental rate, those from the acid origin population seemed to suffer from neutral stress in terms of reduced size at metamorphosis.

Keywords: acidification, adaptive plasticity, amphibians, inducible defences, multiple stressors, stress tolerance.

INTRODUCTION

Phenotypic plasticity is a widespread and important adaptation to spatially and temporally varying environments (Stearns, 1992; Pigliucci, 2001). Plastic responses to single environmental

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factors have received much attention (Pigliucci, 2001), but recent studies show that the expression of plasticity can be affected by interactions between different environmental factors (Laurila and Kujasalo, 1999; Glynn *et al.*, 2003; Lass and Spaak, 2003; Rundle *et al.*, 2004): some factors may trigger antagonist adaptive responses, while additional stressors can either decrease investment in an adaptive plastic response or increase the costs of this response. Understanding environmental dependence of adaptive plastic responses is particularly important for contemporary populations, because these are exposed to an increasing number and amount of novel environmental stressors (Sih *et al.*, 2004). However, the effects of additional stressors on the expression of adaptive plasticity have remained under-explored.

Inducible defences are an important means of anti-predator defences in many organisms, and are predicted to incur costs that prevent their constitutive expression (Tollrian and Harvell, 1999). As predators are ubiquitous in nature, any environmental factor that impairs the expression of inducible defences may have negative effects on individual fitness, which then could lead to major consequences at the population level. Yet, very little is known about how inducible defences are expressed in the presence of additional stressors.

Environmental changes may have many direct and interactive negative effects on contemporary populations (e.g. Kiesecker *et al.*, 2001; Relyea and Mills, 2001). These effects arise in part because organisms experience stress from sources (e.g. global warming; pesticides) that add to stress already occurring normally. These effects are likely to be more severe when organisms have no selective history with these stressors ('novel stressors').

Environmental stressors can influence adaptive responses both because of the cumulative costs of adaptive responses and because of decreased overall condition due to a stressful environment (Petterson and Brönmark, 1997; Hanazato, 2001; Christin *et al.*, 2004; Huber *et al.*, 2004). In this context, additional stressors can decrease the ability to express adaptive plasticity (e.g. Barry, 2000; Relyea, 2004; Teplitsky *et al.*, 2005), such as inducible defences. The sensitivity of adaptive plasticity to these additional stressors may depend on the identity of the stressor and the population. Because environmental stressors can impose strong selection on organisms (Reznick and Ghalambor, 2001) and lead to rapid evolution, local adaptation may overcome the negative effects of the stressors.

We investigated the effects of environmental stress (acidity) on the expression of inducible defences in two moor frog (*Rana arvalis*) populations originating from localities with contrasting acidities. Differences in acidity arise because while wide areas have been severely acidified during the past hundred years due to human activities, some areas have remained neutral due to better buffering capacity of the soil. We chose this system because coping with acid stress is energetically demanding (Dockray *et al.*, 1996) and because previous work found that, at least during the embryonic stage, acid origin populations have higher acid-stress tolerance than do neutral origin populations (e.g. Räsänen *et al.*, 2003). These factors suggest that (i) acid stress could compromise the ability of tadpoles to invest in inducible defences, but that (ii) this effect might differ among populations. In *R. arvalis*, inducible defences consist of modifications of morphology [typically deeper tails and shorter bodies (McCollum and Van Buskirk, 1996; McCollum and Leimberger, 1997; Van Buskirk *et al.*, 1997)] that incur some costs [e.g. competitive ability, reduced growth and development rates (McCollum and Van Buskirk, 1996; Van Buskirk, 2000)], thereby setting the stage for potential trade-offs between responses to acid stress and responses to predators.

We predicted that acid stress could impair the expression or increase the cost of investing in inducible defences. However, because acidity is a novel stressor for the neutral origin population (NOP), we predicted that the performance of NOP tadpoles should be more

impaired by acid stress than that of tadpoles from the acid origin population (AOP). In turn, whereas acidity should have little impact on the fitness of AOP tadpoles, their higher acid tolerance may trade-off with their ability to display inducible defences.

MATERIAL AND METHODS

Experimental protocol

Rana arvalis is widely distributed across the Palearctic. It occurs in various types of habitats at a broad range of pH values and is the most acid-tolerant amphibian species in Europe (Andrén *et al.*, 1988). The acid origin population (Tottatjärn 57°36′N, 12°34′E, mean pH = 4.2) is within an area in south-western Sweden that has been severely acidified due to acid rain (Renberg *et al.*, 1993), whereas the neutral origin population (Häggedal 59°52′N, 17°14′E, mean pH = 7.5) is within an area of central Sweden characterized by calcareous soil and neutral pH (Brunberg and Blomqvist, 2001). Large insect predators (e.g. such as diving beetles *Dytiscus* sp. and dragonfly larvae *Aeshna* sp.) are abundant at both sites (A. Laurila and K. Räsänen, personal observation). Meteorological data for the general geographic areas (data from the Swedish Meteorological Institute, http://www.smhi.se) suggest that the length of the growing season should be roughly 10 days longer at the lower latitude acid origin population than at the higher latitude neutral origin population. However, the populations typically start breeding at roughly the same time (K. Räsänen *et al.*, personal observation) and the differences in season length are likely to be small.

Ten freshly laid clutches were collected from each population and transported to the laboratory in Uppsala, Central Sweden. Clutches were divided: one-half was exposed to low pH and the other half to neutral pH, according to the pH they would be exposed to later in the experiment. The experiment began when tadpoles reached stage 25 [first feeding stage and gill absorption completed (Gosner, 1960)]. At this point, tadpoles were haphazardly chosen from a mixture of the ten clutches and exposed to a combination of two pH (acid pH ≈ 4.25, neutral pH ≈ 7.5) and two predator (predator present: caged dragonfly larva; predator absent: empty cage) treatments. The experiment was run in eight glass aquaria ($144 \times 53 \times 15$ cm) with two aquaria per predator and pH combination. Each of the two aquaria within a treatment was connected in a circulating water system (volume 300 litres), filled with reconstituted soft water (see Räsänen *et al.*, 2003, for details). Reconstituted soft water has a nominal pH of 7.2–7.6, and was used unadjusted in the neutral treatment. In the acid treatment, pH was adjusted daily to 4.25 with sulphuric acid. To maintain high water quality, about 50% of the water was changed weekly in each treatment. Temperature in the laboratory was maintained at 20°C, and the lighting followed the natural photoperiod.

Each aquarium contained eight tadpole cages (four cages per population) and four predator cages. The tadpole cages were distributed randomly according to population of origin, but so that the distance to the predator cages was the same for all cages. This design did not allow complete independence of each replicate, but was adopted because it allowed us to maintain a stable pH in the acid treatment. Most importantly, within each treatment the conditions were exactly the same for both populations.

The tadpole cages ($22 \times 22 \times 15$ cm) were made of plastic netting and acted as the experimental units. Eight randomly chosen tadpoles from a given population were placed in each unit and fed every day *ad libitum* with lightly boiled chopped spinach. The predator

cages (diameter 8 cm, height 21 cm) were made of transparent plastic film and fine-meshed plastic netting, which allowed the tadpoles to receive both visual and chemical cues from the predators. Late-instar dragonfly larvae of the genus *Aeshna*, collected from neutral ponds around Uppsala, were used as predators. Although the predators were captured from neutral sites, they fed readily on tadpoles under both acid and neutral pH, indicating that predator performance was not impaired by acid conditions. In the predator-present treatment, one dragonfly larva was placed in each cage. In the predator-absent treatment, the predator cages were left empty. The predators were fed 300 mg of live *R. arvalis* tadpoles every other day.

Response variables

We measured tadpole responses at two different stages: first, at day 20 after the start of the experiment; second, when the tadpoles reached metamorphosis. At each time, we sampled tadpoles and preserved them in alcohol for later morphological measurements. To maintain density (at four tadpoles) for the latter part of the experiment, and due to mortality, the number of tadpoles sampled at day 20 ranged from three to four. For each tadpole collected at day 20, we measured five morphological traits involved in inducible defences (e.g. Van Buskirk *et al.*, 1997) with a digital calliper (to the nearest 0.01 mm). These included body length, maximum body depth, tail length, maximum tail fin depth, and maximum tail muscle depth.

When the tadpoles approached metamorphosis, the cages were checked daily. At metamorphosis [appearance of the first forelimb, stage 42 (Gosner, 1960)], we recorded body mass and length of the larval period (days elapsed from the start of the experiment) for each individual.

Experimental design and statistical analysis

The experimental design was a $2 \times 2 \times 2$ factorial design with population (acid origin population or neutral origin population), predator presence (absent or present), and pH (acid or neutral) as factors. There were eight replicates in each treatment combination resulting in 64 experimental units. Cages (and hence population and its interactions) were nested within aquarium and aquarium was nested within the predator $\times$ pH interaction. To take this design into account, population of origin and interaction between population and experimental factors were tested using variation among cages as an error term. Other effects (predator, pH, and their interaction) were tested over the among-aquaria effect. For simplicity, cage and aquarium effects are not reported in the tables. In the morphological analyses of tadpoles, tadpole size was first estimated for both populations simultaneously from the first axis of a principal components analysis (PC1) performed on the five morphological variables. Size effects on morphological variables were then controlled for by analysis of covariance (ANCOVA) using PC1 as a covariate. Multivariate analysis of covariance (MANCOVA) followed by univariate ANCOVAs was used to analyse variation in morphology. There was no significant interaction between the covariate and the other factors. Because of lack of degrees of freedom, the predator, pH, and predator $\times$ pH effects could not be estimated in the MANCOVA. Consequently, only the population effect and its interactions between the experimental treatments were estimated in MANCOVA and are reported in Table 1.

Mass at metamorphosis and length of the larval period were analysed with multivariate analysis of variance (MANOVA) followed by univariate analysis of variance (ANOVAs). Tadpole mortality was analysed with type III general linear models with a logit link function and binomial error structure as implemented in the GENMOD procedure of SAS. However, survival was not affected by experimental treatments (all $P > 0.15$) and is not reported further here. All analyses were conducted with the SAS V8 statistical package (SAS Institute, 1999).

RESULTS

Tadpole morphology

In the presence of predators, tadpoles had a deeper tail fin and tail muscle (Table 1, Fig.1), and a shorter body, than in the absence of predators (Table 1). However, the populations responded differently to pH and predator presence, as indicated by the population × predator × pH interaction in the MANCOVA (Table 1). This result arose because the acid origin population expressed the predator-induced phenotype irrespective of pH, whereas the neutral origin population expressed the predator-induced phenotype only in the neutral treatment (Fig. 1). Variation in tail-fin depth was largely responsible for the difference (Fig. 1a, Table 1).

Metamorphic traits

In both populations, predator presence resulted in later metamorphosis at a larger size (Table 2, Fig. 2a). In contrast, pH affected the metamorphic traits differently in the two populations. Exposure to the acid treatment did not affect metamorphic size in the neutral origin population, whereas it resulted in increased size in the acid origin population. Moreover, metamorphosis was delayed in the acid treatment in the neutral origin population, but not in the acid origin population. In the neutral origin population, this resulted in an additive effect of acidity and predation stress on age at metamorphosis: in the acid/predator-present treatment, tadpoles metamorphosed on average 6 days (17.6%) later than in the neutral/predator-absent treatment (Fig. 2b). Tadpoles of the acid origin population performed better in the acid than in the neutral treatment: they had an equally long larval period in both treatments but metamorphosed at larger size in the acid treatment (Fig. 2a,b).

DISCUSSION

We investigated the combined effects of acid stress and predator presence on inducible defences in two *R. arvalis* populations originating from contrasting acidities. Our results show that an additional environmental stressor, in this case acidity, can impair the expression of adaptive plasticity (e.g. inducible defences). However, we also found that populations differed in their responses – possibly depending on the extent to which natural selection has overcome the negative effects of acid stress as a result of local adaptation. However, as we only had one acid and one neutral population in this study, even tentative inferences about local adaptation need to be made with great caution. Nevertheless, we wish to emphasize some important points that should be explored in future work.

Table 1. Results of MANCOVA and univariate ANCOVAs of morphological responses of *Rana arvalis* tadpoles to predator presence and pH in two populations originating from contrasting acidity

Source:	MANCOVA				Tail-fin depth		Tail length		Muscle depth		Body length		Body depth	
Variable	d.f.	*F*	*P*	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Size	5, 225	1127.99	**<0.001**	1, 225	587.68	**<0.001**	340.09	**<0.001**	336.34	**<0.001**	543.79	**<0.001**	252.56	**<0.001**
Population	5, 2	64.07	**0.015**	1, 6	40.85	**<0.001**	0.05	0.826	6.98	**0.038**	0.13	0.735	4.50	**0.078**
Predator	—	—	—	1, 3	21.76	**0.019**	1.51	0.307	33.88	**0.010**	44.54	**0.007**	0.37	0.585
pH	—	—	—	1, 3	1.84	0.268	5.18	0.107	1.94	0.258	0.07	0.803	0.78	0.441
Pop × Pred	5, 2	4.39	0.196	1, 6	2.70	0.151	0.41	0.547	0.12	0.744	0.69	0.439	0.18	0.688
Pop × pH	5, 2	6.30	0.143	1, 6	0.59	0.470	0.04	0.844	3.81	0.099	0.44	0.530	0.11	0.754
Pred × pH	—	—	—	1, 3	0.52	0.522	0.70	0.463	10.47	**0.048**	1.94	0.258	0.02	0.905
Pop × Pred × pH	5, 2	12.89	**0.073**	1, 6	16.21	**0.007**	0.11	0.750	0.00	0.963	0.70	0.434	0.84	0.393

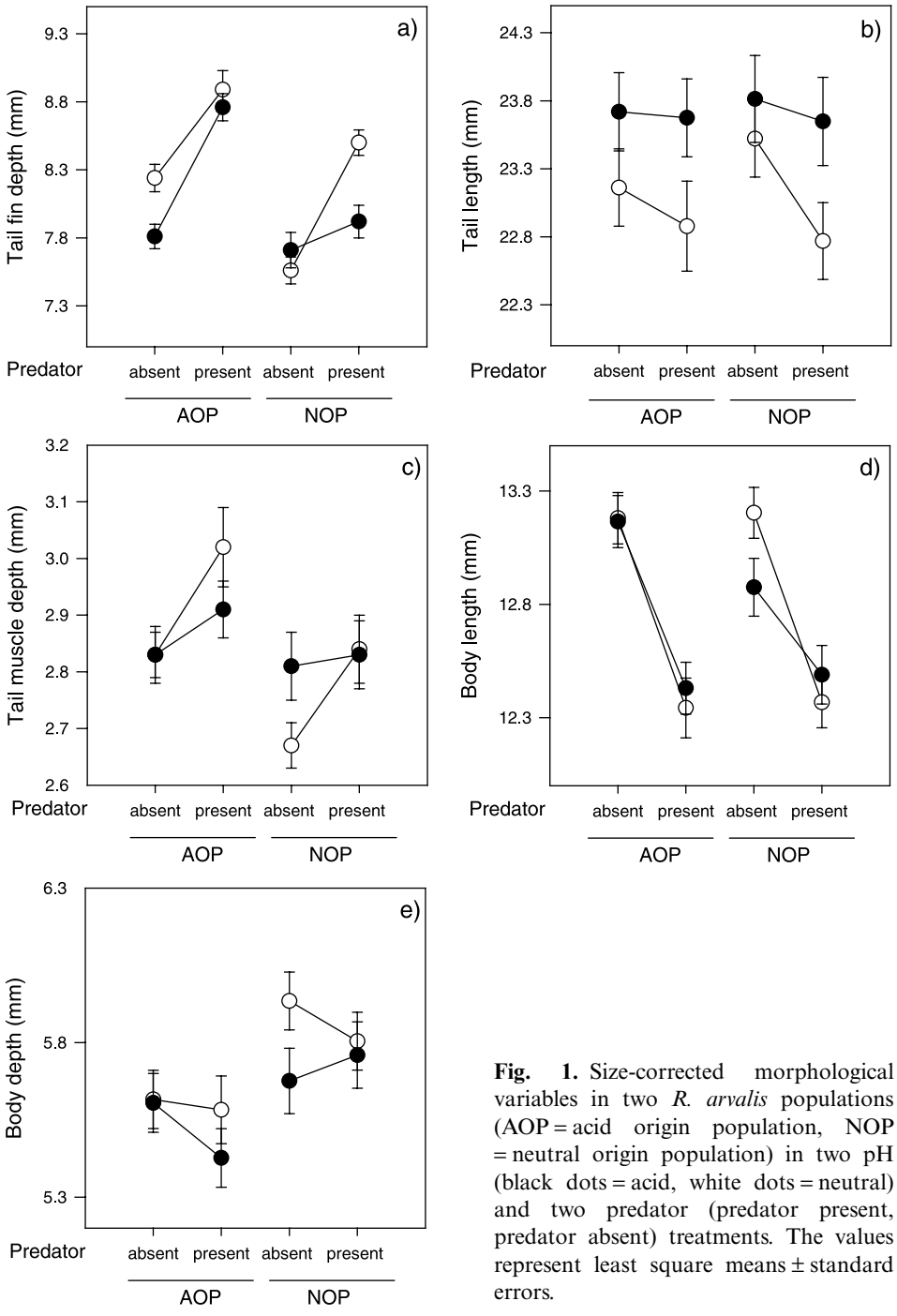

Fig. 1. Size-corrected morphological variables in two *R. arvalis* populations (AOP = acid origin population, NOP = neutral origin population) in two pH (black dots = acid, white dots = neutral) and two predator (predator present, predator absent) treatments. The values represent least square means ± standard errors.

Table 2. Results of MANOVA and ANOVAs of life-history traits of *R. arvalis* metamorphs in response to predator presence and pH

Source:	MANOVA			Mass			Age	
	d.f.	*F*	*P*	d.f.	*F*	*P*	*F*	*P*
Population	2, 5	19.60	**0.004**	1, 6	8.81	**0.025**	24.73	**0.003**
Predator	2, 2	43.77	**0.022**	1, 3	2.78	0.194	129.61	**0.001**
pH	2, 2	25.98	**0.037**	1, 3	1.60	0.295	76.97	**0.003**
Pop × Pred	2, 5	1.36	0.338	1, 6	2.28	0.182	0.23	0.651
Pop × pH	2, 5	8.82	0.023	1, 6	7.07	**0.038**	19.28	**0.005**
Pred × pH	2, 2	0.40	0.716	1, 3	0.15	0.724	1.06	0.379
Pop × Pred × pH	2, 5	1.60	0.290	1, 6	1.21	0.322	1.46	0.273

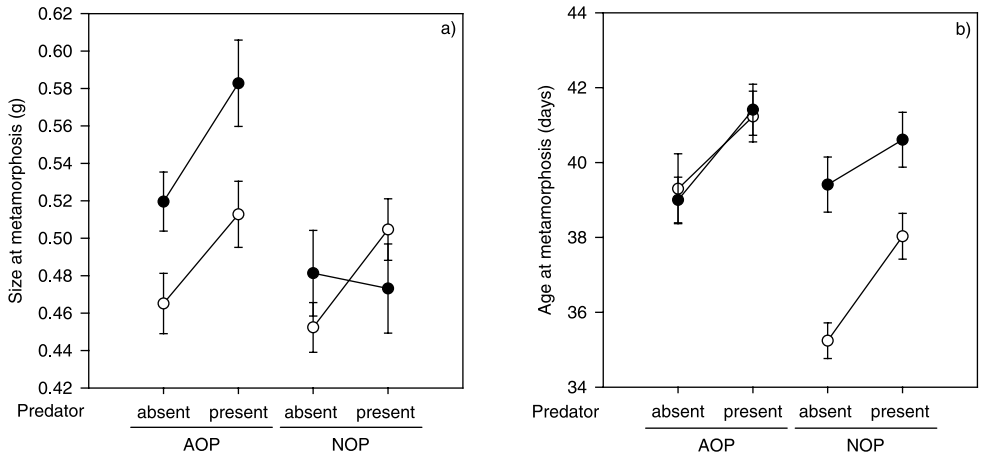

Fig. 2. Mass (a) and age at metamorphosis (b) in two *R. arvalis* populations (see Fig. 1 for caption details). The values represent least square means ± standard errors.

Acidity affected the expression of inducible defences in the neutral origin population by reducing the investment in defence: NOP tadpoles did not develop deeper tails in the presence of predators when simultaneously challenged with acid stress. Because tail fin depth is the main component of the morphological defences in amphibians, decreased investment in this trait could result in reduced survival during predator encounters (Van Buskirk *et al.*, 1997; Van Buskirk and Arioli, 2002). Such inhibitory effects of environmental stress on inducible defences could have strong fitness consequences in taxa where inducible defences play a major role in adaptation to environmental variation. In studies of adaptive plasticity, organisms are often exposed to relatively benign conditions and a single stress factor, which allows them to express the adaptive phenotype in response to the stressor of interest. Our results, together with other lines of evidence (Petterson and Brönmark, 1997; Van Buskirk, 2000; Koricheva, 2002), suggest however that the impact of additional stressors needs to be considered if we are to understand how phenotypic plasticity affects fitness in natural populations.

We found evidence for costs of inducible defences in both populations. Both populations had longer development time in the presence of predators. In amphibians and many other organisms, longer larval development is coupled with larger size at metamorphosis (Wilbur and Collins, 1973). However, although tadpoles developed more slowy in the presence of a predator, this did not lead to a significantly increased metamorphic size. A prolonged larval period coupled with relatively small size could impair fitness because late metamorphosis at small size may reduce juvenile survival and adult fecundity (Smith, 1987; Berven, 1990; Goater, 1994; Altwegg and Reyer, 2003). While other additional stressors, such as pesticides, have already been shown to increase the costs or decrease the investment in defence (Barry, 2000; Schulz and Dabrowski, 2001; Teplitsky *et al.*, 2005), our results show only additive effects of acidity on the costs of inducible defence. This is consistent with the low investment in defence in the neutral origin population at low pH.

The populations seemed to express the costs of pH-related stress differently: the acid origin population had a reduced metamorphic size under neutral conditions, whereas the neutral origin population developed more slowly under acid conditions. These results agree with our expectation that acid conditions are more stressful to NOP tadpoles, but they also indicate that neutral conditions can be a stress factor for AOP tadpoles. This would be the case if the populations differ in their acid tolerance range, possibly due to local adaptation. However, the question remains as to why the populations express the costs in different traits (age vs. size). Currently, the reasons for this are unclear and we can only speculate about them. If we assume that the differences are the result of past selection, then perhaps selection on age versus size, as well as the mechanisms involved, differ between the populations. Selection on age and size at metamorphosis in amphibians often arises via time constraints related to season length (Rowe and Ludwig, 1991; Newman, 1992). In the current study populations, the differences in season length *per se* are likely to be small (see Methods) or, if anything, suggest that the acid origin population should be slightly less time constrained (which would relax selection for development rate). The clearest difference between the populations that we are currently aware of relate to acidity: acid environments are physiologically very stressful, slow-growth environments that typically delay development and reduce metamorphic size [*R. arvalis* (this study; Räsänen *et al.*, 2005), other amphibian species (Räsänen and Green, in press)]. It is possible that these differences have led to genetic differences in larval life-history strategies and their physiological bases in those populations. Finally, the effects of larval growth conditions on age and size at metamorphosis can have carry-over effects on the fitness of the terrestrial stages (Altwegg and Reyer, 2003). At this point, however, we are left with hypotheses; this question needs to be addressed in future research.

Because only two populations were used in this study, the following arguments certainly require further investigation. Nevertheless, we would like to point out that the differences in origin of the two populations were consistent with their different sensitivities to acid stress, both in life-history traits and ability to respond to predator presence. First, under acid conditions, AOP tadpoles were able to display more extensive inducible defences than NOP tadpoles. Second, AOP tadpoles were largest at metamorphosis in the acid/predator-present treatment, suggesting highest fitness under these conditions. These results imply that the ability to express adaptive traits under stressful conditions could partly depend upon identity of the stressor: a stressor may have no negative impact on the expression of adaptive plasticity in a population that has been exposed to directional selection for higher stress tolerance, compared with a population that has not previously encountered the

stressor. This interpretation is further supported by our previous studies on these and other *R. arvalis* populations showing adaptation to acidity in embryonic survival, and egg-size effects on larval life histories (Räsänen *et al.*, 2003, 2005).

Several examples support the theory that ecological and evolutionary responses of populations to anthropogenic changes may strongly depend on interactions with other abiotic or biotic factors. For instance, tolerance to one stressor may trade off with the ability to cope with other stressors [e.g. trade-off between insecticide resistance and higher burdens of parasitic infection (Berticat *et al.*, 2002)]. In the same vein, stress tolerance could trade off with adaptive plasticity. While studies on multiple populations will be needed to test the contribution of local adaptation to among-population variation in the stress responses observed in the current study, our results suggest higher acid tolerance, and no trade-off between acid tolerance and adaptive responses to predators in the acid origin population. At least that is a hint that natural selection can lead to adaptation to multiple stressors.

In summary, our study shows that inducible defences may be constrained in the presence of environmental stressors but that the extent of these effects differs among populations. While the use of a limited number of populations in this study prevents us from a conclusive answer to the question about how local adaptation to one stressor may influence responses to additional stressors, the results highlight the need for future studies to include multiple stressors and multiple populations when investigating the costs and benefits of adaptive plasticity. Moreover, such constraints on the expression of adaptive plasticity can have strong implications for the fitness of natural populations – particularly when these are exposed to anthropogenic environmental changes. We hope that our results will stimulate research in this exciting area, and that a future meta-analysis can provide a better understanding of the effects of multiple stressors on adaptive plasticity.

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