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Geographic variation in corticosterone response to chronic predator stress in tadpoles

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Abstract

Chronic stress often affects growth and development negatively, and these effects are often mediated via glucocorticoid hormones, which elevate during stress. We investigated latitudinal variation in corticosterone (CORT) response to chronic predator stress in *Rana temporaria* tadpoles along a 1500-km latitudinal cline in Sweden tadpoles, in a laboratory experiment. We hypothesized that more time-constrained high-latitude populations have evolved a lower CORT response to chronic stress to maintain higher growth under stressful conditions. Southern tadpoles had higher CORT content in response to predators after 1 day of exposure, whereas there was no increase in CORT in the northern populations. Two weeks later, there were no predator-induced CORT elevations. Artificially elevated CORT levels strongly decreased growth, development and survival in both northern and southern tadpoles. We suggest that the lower CORT response in high-latitude populations can be connected with avoidance of CORT-mediated reduction in growth and development, but also discuss other possible explanations.

Introduction

Widespread organisms that are exposed to large-scale variation in environmental conditions often exhibit adaptive geographic variation in fitness-related traits (Endler, 1977). For example, adaptive clines in life histories evolve in response to climatic variation along latitudinal and altitudinal gradients (Angilletta 2009, Conover *et al.*, 2009). However, physiological variation underlying adaptive clines has remained much less studied (Feder *et al.*, 2000), and for example, variation in hormonal mechanisms along geographic gradients remains largely unexplored (but see, e.g. Silverin *et al.*, 1997; Hau *et al.*, 2010). Glucocorticoid hormones (GCs, corticosterone or cortisol, depending on the taxon), which are released during stressful situations, are particularly interesting in the context of geographic variation of stress responses. GCs upregulate behaviours essential to addressing potential threats and mobilize available energy by down-regulating functions not essential to immediate survival, such as reproduction, immune function and growth (Orchnik, 1998; Sapolsky *et al.*, 2000). In a short term, a GC response is advantageous as it increases the chances of surviving a life-threatening situation; however, it can have detrimental effects on the future fitness of an individual, particularly when stress situations occur frequently or become chronic (Sapolsky, 2002). Hence, the GC response, initially evolved to survive stressors, can also contribute negatively to many fitness components, such as reproductive output, growth and developmental rate (e.g. Moore & Jessop, 2003; Ellis *et al.*, 2006). Consequently, when severity of a stressor varies geographically, we may expect to find variation in the strength of the stress response (Dunlap & Wingfield, 1995; Silverin *et al.*, 1997).

High-latitude environments are characterized by a short growth season and long winter, and ectothermic animals living under such stringent conditions commonly exhibit higher growth and developmental rates ensuring completion of growth and development during the short vegetative season (Conover *et al.*, 2009).

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A stress response affecting growth and development should be particularly costly to high-latitude populations. Field measurements in birds show that populations breeding at high latitudes exhibit a lower corticosterone (CORT) response to acute handling stress compared to birds breeding in milder climates (Silverin *et al.*, 1997); however, it is not known whether these differences were genetically based or a result of environmental effects.

For most organisms, predation risk is ubiquitous, and prey has evolved a number of ways to minimize the risk of being eaten. A large number of studies have described predator-induced changes in prey behaviour, physiology, life history and morphology (Kats & Dill, 1998; Lima, 1998; Tollrian & Dodson, 1999). However, although these changes reduce predation risk, they also incur costs in terms of reduced growth, reproduction and survival (Lima, 1998; Werner & Peacor, 2003; Preisser *et al.*, 2005). At the hormonal level, predation threat causes a stress response including elevated GC levels in many vertebrate species (e.g. Blanchard *et al.*, 1998; Boonstra *et al.*, 1998; Hubbs *et al.*, 2000; Cockrem & Silverin, 2002; Figueiredo *et al.*, 2003; Thaker *et al.*, 2009a). On the other hand, experimentally elevated GC levels can reduce foraging rates (Gregory & Wood, 1999; but see, e.g. Lõhmus *et al.*, 2006), decrease growth and developmental rates (Barton *et al.*, 1987; Hayes & Wu, 1995; Glennemeier & Denver, 2002; Belden *et al.*, 2005) and increase predator defence behaviours (Kalynchuk *et al.*, 2004; Thaker *et al.*, 2009b). Hence, GCs may prove to be of importance in mediating the behavioural responses as well as the costs associated with predation stress (Lima, 1998).

Individual variation in the magnitude of the GC response is large (Ellis *et al.*, 2006), and individuals often consistently exhibit a high or a low GC response (Pottinger *et al.*, 1992; Tort *et al.*, 2001; Cockrem, 2007). This tendency appears to be heritable (Pottinger & Moran, 1994; Evans *et al.*, 2006), and the costs and benefits of having a low or high response may depend on the type of environment the organisms encounter (Moore & Jessop, 2003; Cockrem, 2007). Hence, it seems possible that populations may show different levels of GC response depending on their habitat. Studies comparing natural populations in the field support this hypothesis (Dunlap & Wingfield, 1995; Silverin *et al.*, 1997; Mateo, 2006). There is also evidence for intraspecific variation in stress-related responses under common garden conditions (Dunlap & Wingfield, 1995; Bell, 2005; Brown *et al.*, 2005; Angelier *et al.*, 2011), which are hypothesized to be adaptive and linked with divergence in behaviour or life history. However, only two studies have compared GC expression in wild populations under common garden conditions (Dunlap & Wingfield, 1995; Angelier *et al.*, 2011).

In amphibians, CORT plays a crucial role in the control of metamorphosis (Denver, 2009). There is growing evidence that CORT is directly involved in mediating genotype–environment interactions in tadpoles, for example, by synergizing with thyroid hormone to proximally cause early metamorphosis induced by pond drying (Denver 1998). Elevated CORT may also be involved in the deepening of the tailfin of tadpoles exposed to predator stress (Glennemeier & Denver, 2002; Hossie *et al.*, 2010). On the other hand, external manipulations of corticosterone (CORT, the main GC in amphibians) in premetamorphic tadpoles have shown that elevated CORT levels decrease growth and developmental rates (Hayes & Wu, 1995; Glennemeier & Denver, 2002; Belden *et al.*, 2005; Ledón-Rettig *et al.*, 2009). Pond-living tadpoles are often chronically exposed to predator stress, and if predator exposure results in a chronic CORT elevation, it may have a negative effect on growth and development in premetamorphic tadpoles.

In Scandinavia, the common frog *Rana temporaria* exhibits a latitudinal pattern with increasing larval growth and developmental rates towards higher latitudes (e.g. Merilä *et al.*, 2000; Laugen *et al.*, 2003; Lindgren & Laurila, 2009). High-latitude tadpoles are more active (i.e. higher rates of swimming and foraging) than low-latitude ones in the presence of predators, and this increases mortality due to predation (Laurila *et al.*, 2008). Large body size and timely completion of development are crucial determinants of juvenile overwintering success in ectotherms (e.g. Smith, 1987; Nagle *et al.*, 2000; Altwegg & Reyer, 2003), and these traits may be especially important at high latitudes (Munch & Conover, 2003; Sears, 2005). As high growth and developmental rates are more crucial in high-latitude populations, we could expect a reduced chronic CORT response in these populations.

In this study, we investigated clinal variation in predator-induced CORT response as well as the effects of external CORT on growth and development in *R. temporaria* in two laboratory experiments. In the first experiment, we measured the CORT response to chronic predator stress under common garden conditions in tadpoles from eight populations along the 1800-km latitudinal gradient across Sweden. We asked: (i) Is there a latitudinal pattern in CORT expression? (ii) Do *R. temporaria* tadpoles respond to predation risk with elevated CORT response. Specifically, we predicted that (iii) high-latitude tadpoles should show a weaker CORT response to avoid costs associated with elevated CORT levels. In the second experiment, we manipulated the external CORT levels in one high- and one low-latitude population, recorded tadpole growth and development and asked the following questions: (iv) Does externally administered CORT affect growth and development of *R. temporaria* tadpoles? (v) Are there differences between populations in sensitivity to elevated CORT levels in terms of growth and development?

Methods

Rana temporaria is the most widespread amphibian in Europe, occurring from northern Spain to northernmost Norway (Gasc *et al.*, 1997). It breeds in a variety of freshwater habitats from temporary ponds to shore marshes of large lakes, and at high latitudes, it is often the only amphibian species present.

Experiment 1: Latitudinal variation in CORT levels and predator-induced CORT response

We collected freshly laid eggs of *R. temporaria* from eight populations along the latitudinal gradient across Sweden (Fig. 1). At each locality, we sampled ca. 500 eggs from each of ten clutches. The eggs were transported to the laboratory in Uppsala, and each egg clutch was distributed evenly over two 3-L buckets. Throughout the study, the eggs and tadpoles were kept in a 19 °C temperature-controlled room under 16-h light/8-h dark photoperiod. To ensure homogenous water quality, we used reconstituted soft water (RSW; APHA, 1985). Water was changed every 3 days before the start of the experiments and every 7 days during the experiment. After hatching, tadpoles were fed *ad libitum* with finely chopped and lightly boiled spinach. Dragonfly larvae of the genus *Aeshna*, collected from ponds near Uppsala, were used as predators as they are voracious predators of tadpoles and occur throughout Scandinavia (e.g. Laurila *et al.*, 2008).

The experiment was a full-factorial, randomized block design with eight populations and two predator treatments (absence or presence). Each treatment combination was replicated ten times. To account for the known temperature gradient within the laboratory room, the containers were divided into five blocks, one for each shelf level. Each block contained two replicates of each treatment combination. We used opaque plastic containers (38 × 28 × 13 cm) as experimental units. Each container was filled with 10 L of RSW and provided with cylindrical transparent mesh-bottom predator cage (diameter 11 cm, height 21) hung 2 cm above the container bottom.

When tadpoles had reached Gosner stage 25 (complete gill absorption; Gosner, 1960), ca. 50 seemingly healthy tadpoles from each family in a population were pooled in a bucket and randomly divided into groups of 20 individuals that were allocated to the experimental containers. One *Aeshna* larva was placed in the predator cage in the containers assigned to the predator treatment (day 0 of the experiment). In the control containers, the cage remained empty. Note that due to the differences in phenology (Fig. 1), the start of the experiment varied among the populations. The predators were fed one *R. temporaria* tadpole daily. Feeding occurred inside the predator cage, so that tadpoles could receive both visual and chemical cues from the predation events.

Fig. 1 Map of Fennoscandia showing the locations of the *Rana temporaria* populations used in this study. Coordinates and collection date for each locality were as follows: *Karesuando* 68° 27′N, 22° 25′E, 6 June 2007; *Björkliden* 68° 24′N, 18° 41′E, 24 May 2006; *Jukkasjärvi* 67° 54′N, 21° 02′E, 23 May 2006; *Holmsjön* 63° 58′N, 19° 34′E, 8 May 2006; *Mjösjön* 63° 58′N, 20° 25′E, 8 May 2006; *Nordsmyran* 60° 35′N, 17° 12′E, 2 May 2006; *Uppsala* 59° 51′N, 17° 28′E, 27 April 2006 and 1 April 2007; *Tvedöra* 55° 41′N, 13° 26′E, 18 April 2006; *Ållskog* 55° 33′, 13° 48′E, 18 April 2006.

On day one and day 15 at 9 a.m., one tadpole was removed from each container. On both sampling occasions, the last predator feeding event was ca. 16 h prior to the removal of the tadpoles. The tadpole was placed in a test tube, immediately frozen by dipping the test tube in liquid nitrogen and stored in −80 °C until radioimmunoassay (RIA) (described below). On day 21, the remaining tadpoles were collected and preserved in 70% ethanol. Body size, defined as the length from the snout to the base of the tail, was measured from each individual (body length was used rather than mass, as weight is difficult to interpret for tadpoles preserved in ethanol), and Gosner developmental stage was determined.

Analysis of hormone contents: RIA/TLC

Whole-body CORT content was determined by RIA following the protocol by Licht *et al.* (1983) with modifications. Extractions were performed as described by Hayes & Wu (1995). Samples from the two measuring time points (days one and 15 of the experiment) were assayed separately. Briefly, whole-body homogenates were used for each assay. Each tadpole was homogenized in 2 mL ethyl acetate. For individual recovery

determination, 3000 cpm of triated CORT (Perkin Elmer Product number: NET399250UC) was added to the homogenates. To break emulsion, samples were centrifuged at $1300 \times g$, and the organic phase was then removed and dried under a stream of nitrogen. The extracts were resuspended in ethyl acetate and fractioned by thin-layer chromatography (TLC) to separate CORT from other products, particularly lipids. The region of the gel containing the CORT was scraped and the silica collected and extracted with ethyl acetate. The extract was dried and resuspended in 0.5 mL phosphate-buffered saline containing 0.1% gelatin (PBS-G). Samples with recoveries below 40% were removed, and samples used for further analysis had recoveries ranging between 40% and 78% (average 61%).

We used Sigma-Aldrich antiserum (product nr: C8784) and Perkin Elmer triated CORT (product number: NET399250UC). Cross-reactivity of the antiserum was 4.4% for aldosterone, 2.6% for androstenedione, 4.5% for cortisol, 3.2% for cortisone, <10% for dehydroepiandosterone, <20% for 11-deoxycorticosterone, 1.4% for 5α-dihydrotestosterone, <30% estradiol, 1.8% for 17-hydroxyprogesterone, 8.8% for 20α-hydroxyprogesterone, 5.2% for 20β-hydroxyprogesterone, 15.7% for progesterone and 7.9% for testosterone (according to manufacturer). As gonads have not differentiated and are not active in tadpoles, cross-reactivities with estradiol, testosterone, progesterone and their derivatives are negligible. Standards (0–4000 pg mL^{-1}) were run in duplicates, one at the start and another at the end of the assay. For the assay, 400 μL of sample or standard was incubated in 37 °C for 3 h with 50 μL triated CORT (10K cpm) and 50 μL diluted antiserum (15 pg). Unbound CORT was separated by adding 100 μL of dextran-coated charcoal, and bound CORT was decanted into scintillation vials. Samples were run in five assays for each measuring series (days one and 15) and were run blockwise, so that each assay contained an equal number of samples from each treatment and population. Within each block, samples were run in random order. One block was excluded from each measuring series due to very low values (below 0) for all samples (presumably due to unsuccessful extractions); thus, only eight of the ten replicates were used for the final analyses. Intra-assay variation was 6.1% (based on the cpm values of the duplicates of the standards). Inter-assay variation was not calculated, but the block design allowed this to be taken into account in the statistical analysis.

Experiment 2: CORT manipulation

We collected ca. 300 freshly laid *R. temporaria* eggs in each of seven clutches from one population in mid-southern Sweden (Uppsala) and from one population in northern Sweden (Karesuando; Fig. 1). The eggs were transported to the laboratory in Uppsala, and each family was distributed evenly in two 3-L buckets. Water was changed every 3 days prior to the experiment and every 4 days during the experimental period. Tadpoles were exposed to the same water quality, temperature, photoperiod and feeding conditions as in the first experiment.

The experiment consisted of four treatments: control, vehicle (ethanol only), 100 nM of CORT and 500 nM of CORT. Treatments were begun when the tadpoles had reached Gosner stage 25. Ten tadpoles were placed in each plastic container ($38 \times 28 \times 13$ cm) filled with 10L RSW. We had ten replicate tanks in each treatment combination (80 tanks in total). CORT (Sigma-Aldrich, Inc., Saint Louis, MO, USA; Product No. C2505) was dissolved in ethanol and administered to water to give water concentrations of 100 or 500 nM CORT. These levels were chosen based on previous studies using exogenous CORT on tadpoles (Glennemeier & Denver, 2002; Belden *et al.*, 2005). The ethanol concentration in the water was 0.00001%. Vehicle-treated tanks received ethanol only, and control tanks received no treatment. CORT or ethanol was added to the tanks every 4 days in conjunction with each water change.

After 21 days of treatment (and 3 days after the most recent CORT addition), five tadpoles (each from separate container) were removed from each treatment, rapidly rinsed in water and frozen for CORT measurements. Whole-body CORT contents of these individuals were measured as explained above. Once tadpoles were approaching metamorphosis, the containers were checked daily. Metamorphosed individuals (Gosner stage 42; emergence of forelimbs) were removed and weighed to the nearest 0.1 mg, and the length of larval period was recorded.

Statistical analysis

In the predator experiment, size and development data were missing from six of the containers as the samples were destroyed during the storage. We used tank-average of body length and developmental stage as response variables. Mortality was low (4.3%), did not differ among the treatments ($P > 0.05$) and did not affect size or developmental stage at day 21 ($P > 0.05$).

Latitudinal patterns in CORT were analysed using a mixed model ANCOVA with latitude as a covariate, population and RIA block as random effects and predator treatment as a fixed effect. The spatial block did not affect CORT levels and was removed from the analyses. Body length and developmental stage were analysed using a mixed model ANCOVA with latitude as a covariate, population as random effect and predator treatment and spatial block as fixed effects. We used REML estimation for random effects and Type III sums of squares for fixed effects as implemented in Proc Mixed in SAS v 9.2 (SAS institute, Inc., Cary, NC, USA). The data from the CORT manipulation experiment were analysed with general linear models (GLM) in SPSS 19 (IBM SPSS Statistics, Chicago, IL, USA) with population

and hormone treatment as fixed factors. Tadpole survival was analysed as a binomial response variable with generalized linear models in Proc Genmod in SAS using population and hormone treatment as fixed factors.

Results

Experiment 1: Predator experiment

On day one, 24 h after the predators had been introduced, high-latitude tadpoles had lower CORT levels than low-latitude tadpoles ($F_{1,6.05} = 7.40$, $P = 0.034$; Fig. 2a, Appendix S1 for complete analysis). Tadpoles exposed to predators generally had higher CORT levels than those raised in the absence of predators ($F_{1,104} = 5.29$, $P = 0.023$; Fig. 2a). However, predators had a stronger effect in the low-latitude populations as indicated by the significant treatment × latitude interaction effect ($F_{1,104} = 4.46$, $P = 0.037$; Fig. 2a).

At day 15, CORT levels were generally higher than at day one, and there was no significant latitude or population difference in CORT content ($P = 0.158$ for latitude, $P = 0.507$ for population as a fixed effect; Fig. 2b see Appendix S1 for complete analysis), no difference between the predator treatments ($P = 0.792$), nor a significant interaction between latitude and treatment ($P = 0.888$). However, individual CORT levels were negatively correlated with body mass both on day one ($r = -0.39$, $n = 121$, $P < 0.001$) and on day 15 ($r = -0.34$, $n = 115$, $P < 0.001$).

Tadpole body length was positively influenced by latitude ($F_{1,6.04} = 28.49$, $P = 0.002$; Fig. 3a see Appendix S1 for complete analysis). Predator presence did not affect body length ($P = 0.311$; Fig. 4a), and there was no significant treatment × latitude ($P = 0.243$) or treatment × population interaction (fixed effect; $P = 0.372$). High-latitude tadpoles were at a more advanced developmental stage at day 21 ($F_{1,6} = 13.28$, $P < 0.001$; Fig. 3b). Tadpoles raised with a predator were less developed than those raised without a predator ($F_{1,140} = 6.51$, $P = 0.012$; Fig. 3b), and this difference was stronger in the southern populations, as indicated by the significant treatment × latitude interaction ($F_{1,140} = 5.21$, $P = 0.024$).

Experiment 2: CORT Manipulation

21 days of external CORT treatment strongly elevated tadpole whole-body CORT levels ($F_{3,32} = 18.86$, $P < 0.001$; Fig. 4). Tadpole CORT levels were more strongly affected by external CORT in the northern than in the southern population as indicated by the significant treatment × population interaction ($F_{3,32} = 3.18$, $P = 0.037$; Fig. 4), which also resulted in marginally higher CORT levels in the northern population ($F_{1,32} = 3.21$, $P = 0.083$; Fig. 4).

The northern population had significantly shorter larval period than the southern population ($F_{1,72} = 1232.05$, $P < 0.001$; Fig. 5a), and tadpoles in both CORT treatments had longer larval periods than tadpoles in the control treatments ($F_{3,72} = 4.45$, $P = 0.006$). The populations did not differ in their developmental response to the CORT treatments (population × treatment interaction: $P = 0.306$; Fig. 5a). Northern tadpoles were significantly smaller at metamorphosis ($F_{1,72} = 18.78$, $P < 0.001$; Fig. 5b). Tadpoles' body mass was strongly reduced in both CORT treatments as compared to control or vehicle-treated tadpoles ($F_{3,72} = 55.75$, $P < 0.001$; Fig. 5b). There was no significant interaction between the CORT treatments and population origin in terms of mass at metamorphosis ($P = 0.091$). Growth rate was significantly higher in the northern population ($F_{1,72} = 184.33$, $P < 0.001$, Fig. 5c).

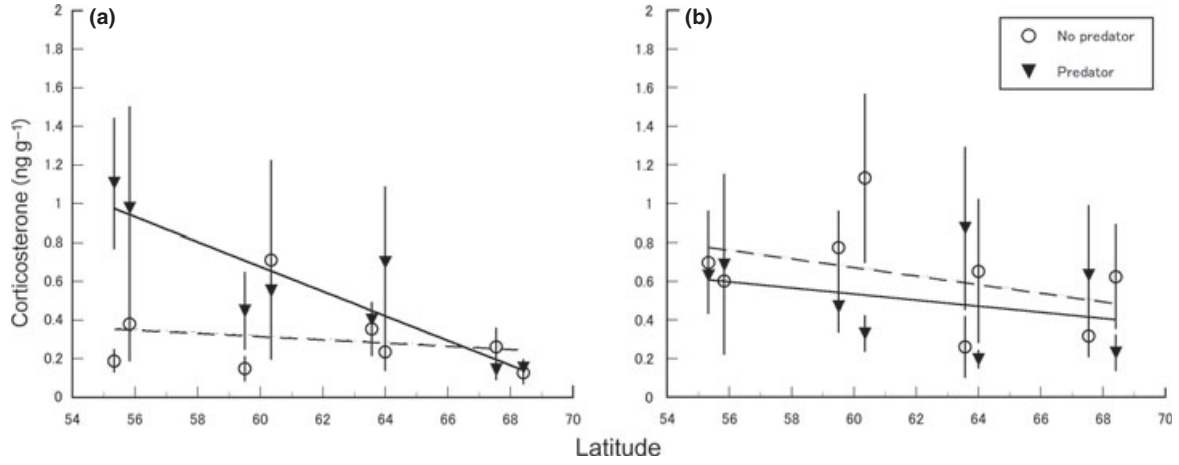

Fig. 2 Mean (±SE) corticosterone content (ng g^{-1}) along the latitudinal gradient in tadpoles raised with (solid line, black triangles) and without (dashed line, open circles) predators after 1 (a) and 15 (b) days of exposure.

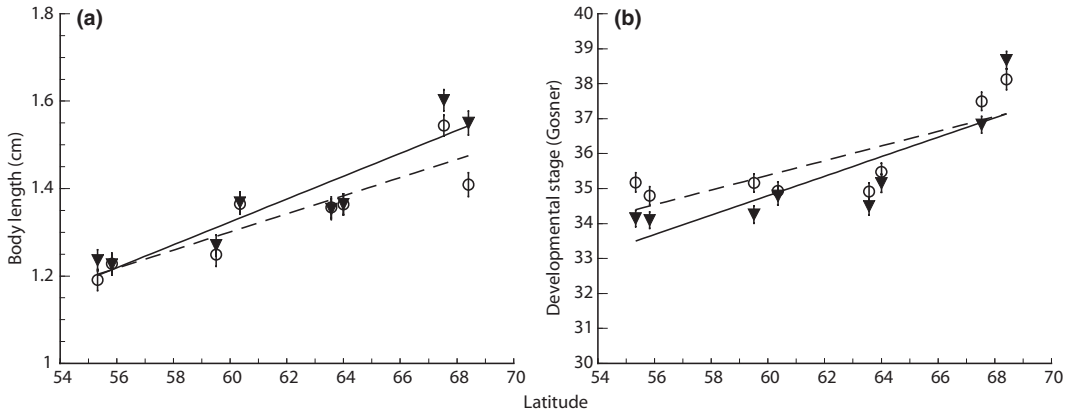

Fig. 3 Mean (±SE) body length (a) and developmental stage (b) along the latitudinal gradient measured on tadpoles after 21 days of predator exposure (predator present: black triangles, solid black line; control: open circles, dashed line).

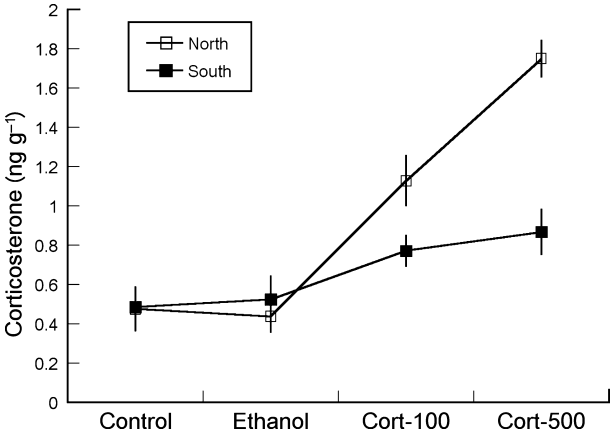

Fig. 4 Mean (±SE) whole-body CORT contents in tadpoles from a northern (open squares) and a mid-southern latitude population (black squares) exposed to control treatment, ethanol vehicle control, 100 nM CORT or 500 nM CORT for 21 days.

CORT-treated tadpoles from both treatments grew much slower than tadpoles from either control treatments ($F_{3,72} = 52.17$, $P < 0.0001$; Fig. 5c). There was no significant interaction between the CORT treatments and population origin ($P = 0.729$).

The higher CORT dose (500 nM) resulted in roughly four times higher tadpole mortality than in the control treatment ($F_{3,72} = 11.88$, $P < 0.001$; Fig. 5d). Ethanol and 100 nM CORT treatments did not significantly affect mortality ($P > 0.05$). There were no significant population ($P = 0.869$) or population × treatment interaction ($P = 0.566$) effects in terms of mortality.

Discussion

We found that *R. temporaria* tadpoles from low-latitude populations exposed to predators for 24 h had elevated whole-body CORT levels, whereas there was no response in high-latitude tadpoles, suggesting genetic or maternal effect variation in predator-induced CORT expression along the latitudinal gradient. This pattern was transient, and no induced responses or a latitudinal cline was found in CORT expression after 15 days of predator exposure. Artificially elevated CORT levels decreased tadpole growth, development and – at higher concentrations – survival. As rapid early growth may have a larger fitness value in time-constrained environments, weaker CORT response in high-latitude populations may be related to the higher adaptive value of rapid growth and development in time-constrained environments.

Predator-induced CORT levels

We found that predator-exposed tadpoles exhibit a CORT response after 24 h of predator exposure. On average, the CORT levels were elevated 1.8-fold as compared to the control treatment; however, the response varied widely among the populations. After 15 days of predator exposure, there was no significant difference between predator-exposed and control tadpoles. Most studies on vertebrates have measured the predator-induced GC levels within minutes to 1 h of exposure and typically found 1.5- to 3.5-fold elevations in plasma concentration of GCs (e.g. Cockrem & Silverin, 2002; Figueiredo *et al.*, 2003; Barcellos *et al.*, 2007; Thaker *et al.*, 2009a). The only study that we are aware of that has investigated predator-induced GC levels in amphibian larvae (two *Rana* species from Michigan, USA) found a decrease in CORT content after 4 h of exposure (no measurements from later time points were reported), which was hypothesized to mediate the decrease in activity associated with predator-stressed tadpoles (Fraker *et al.*, 2009). In the present study, the CORT measurements were made on tadpoles exposed to predator presence for 24 h and 15 days and thus represent chronically stressed tadpoles. While the

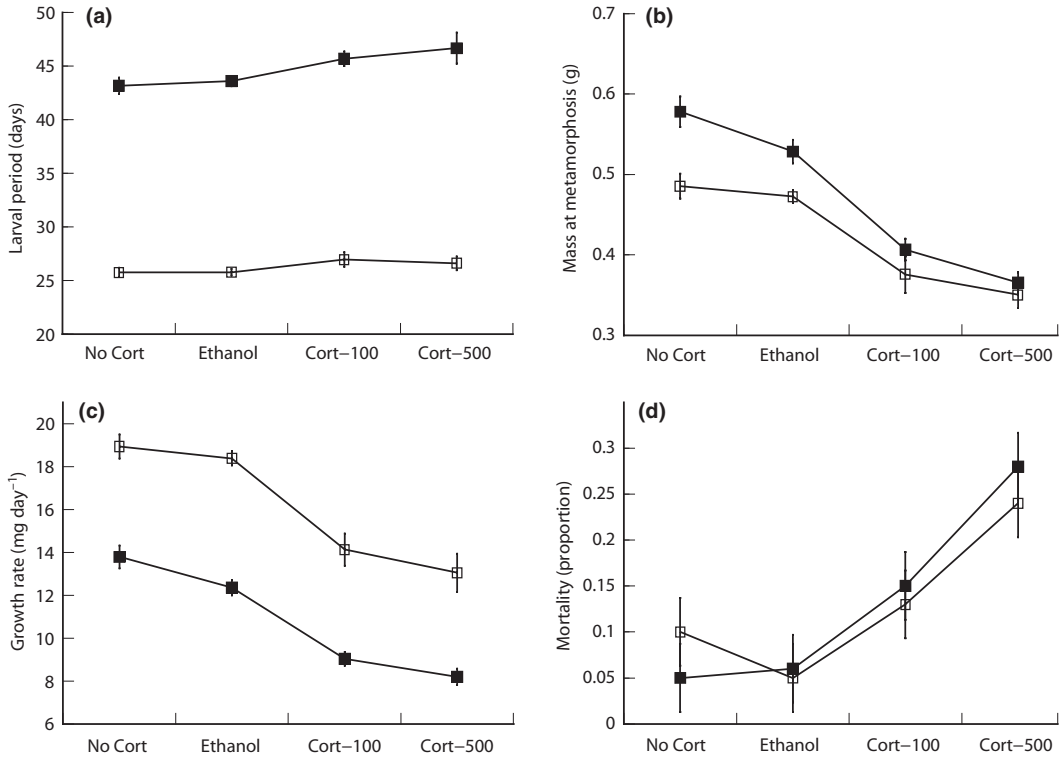

Fig. 5 Mean (±SE) larval period (a), mass at metamorphosis (b), growth rate (mass at metamorphosis per days until metamorphosis) (c) and mortality (d) for tadpoles from northern (open squares) and mid-southern (black squares) Sweden exposed to control treatment, ethanol vehicle control, 100 nM CORT or 500 nM CORT.

decrease in CORT found by Fraker *et al.* (2009) seems to be associated with the initial response (and initiation of the predator-induced suppression of activity), the present study suggests that chronically predator-stressed tadpoles can go through a period of CORT elevation. We chose to study the effects of chronic predator stress, because this is the normal environment experienced by tadpoles in nature (dragonfly larvae and tadpoles coexist in the confined ponds for several weeks) and more likely to be reflected in growth and development rates. This type of predator treatment is also most commonly used in experimental studies of predator effects on amphibian larvae (Relyea, 2007).

Previous studies have found that vertebrates may habituate and down-regulate the GC levels when exposed chronically to mild stressors (captivity and handling; e.g. Barton *et al.*, 1987; Dobrakova & Kvetnansky, 1993). However, the response to predatory stress seems to display minimal or no habituation, and mammals that are repeatedly or chronically exposed to predators continue to exhibit elevated GC (Blanchard *et al.*, 1998; Boonstra *et al.*, 1998). In our study, tadpoles down-regulated CORT elevations within 15 days of chronic predator stress. This could be due to ecological differences among the major vertebrate clades: whereas many mammal species may temporarily escape predation threat, larval amphibians living in confined ponds may have little possibility to recover between predator threat events and have a better capacity to down-regulate their hormonal stress response under prolonged predation threat.

Predator-stressed *R. temporaria* tadpoles display a marked decrease in foraging and activity rates, a behaviour that does not cease with prolonged exposure (e.g. Laurila *et al.*, 2004, 2008; E. Dahl, pers. obs. in this study), indicating that tadpoles do not habituate behaviourally to predator stress and continue to perceive the dragonfly larva as a serious threat. GC levels can be down-regulated by negative feedback where elevated concentrations interact with receptors in the brain to turn off the initial steps of the HPI axis, so that levels of GCs first rise and then fall even when stress continues (Dallman *et al.*, 1992). This is likely to be the case when a chronic stressor ceases to produce a CORT response though other stress-related changes (i.e. increase in glucose levels, behavioural changes) remain and could be a way of avoiding costs of prolonged CORT exposure (Barton *et al.*, 1987). Although no other physiological measurements were made in this experiment, the lack of behavioural habituation to predators in *R. temporaria* suggests that this was probably the case in the present study.

Latitudinal differences

We found that predator-induced CORT levels in tadpoles after 24 h of predator exposure were lower towards higher latitudes, the response varying from a slight decrease in two of the populations (one mid- and one northern) to a 4.2-fold increase in the southernmost population. Intraspecific differences in GC response under common garden conditions have previously only been found in western fence lizards (Dunlap & Wingfield, 1995) where populations near the periphery of the species range showed higher GC response, and in between two subspecies of swamp sparrows where males of the subspecies showing more aggression but lower reproductive effort had higher GC responses (Angelier *et al.*, 2011). The differences in our system could be due to adaptive differences originating from contrasting environmental constraints. One possibility is that the elevated CORT responses may be too costly in terms of growth and development for time-constrained northern tadpoles. The lower CORT response is in accordance with field studies on birds, which have shown that high-latitude populations, which have to complete breeding and moulting in a short time, show a lower CORT response to handling stress (Silverin *et al.*, 1997). In amphibians, time-constrained and fast-developing *Scaphiopus holbrooki* tadpoles lacked the CORT response to confinement stress found in two less time-constrained species (Belden *et al.*, 2010). Alternatively, predator-induced CORT response may also be under weaker selection in northern tadpoles as they are exposed to lower predator densities than low-latitude populations (Laurila *et al.*, 2008). This hypothesis is supported by previous studies where fish populations from high predation regions are more reactive (measured by opercular beat frequency) and more behaviourally responsive when exposed to stress than fish from low predation regions (Brown *et al.*, 2005; Bell *et al.*, 2010).

We also found that basal CORT levels decreased significantly with latitude on day one, and there was an overall negative correlation between body mass and CORT both at days two and 15, a pattern that has been found previously in tadpoles (Belden *et al.*, 2007; Ledón-Rettig *et al.*, 2009). Although stress-induced CORT elevations suppress growth rates, baseline CORT concentrations are mediated by different receptors and play a permissive role in many basic functions of vertebrate physiology (reviewed in Romero, 2004). While it seems that basal CORT levels and growth rate are negatively correlated in several amphibian species, the exact role of basal CORT in determining growth rates remains open.

Our experimental design cannot decipher whether parental environmental effects play a role in our results (e.g. Badyaev & Uller, 2009). Maternal effects appear to be small compared to genetic effects on the latitudinal growth patterns in *R. temporaria* (e.g. Laugen *et al.*, 2002); however, it is not known whether the maternal environment affects offspring CORT levels in amphibians, as has been observed in other vertebrate taxa (Hayward & Wingfield, 2004; Giesing *et al.*, 2011; Michel *et al.*, 2011). Further studies are needed to resolve these issues. Further, the antibody used in this study has rather high cross-reactivities with other glucocorticoids, which also increase with stress (e.g. cortisol and cortisone). However, as the other glucocorticoids are likely to occur in small amounts in amphibians (Idler, 1972), cross-reactivity should result in only a very slight overestimation of CORT levels.

Effects of artificially elevated CORT levels

Prolonged exposure to exogenous CORT resulted in an increase in whole-body CORT in the range of those previously reported for similar doses (e.g. Hayes & Wu, 1995; Glennemeier & Denver, 2002), which in turn are within the range of natural CORT elevations in response to environmental stress (Hayes & Wu, 1995; Denver, 1998; Glennemeier & Denver, 2002). We found that northern tadpoles in the CORT treatments had higher contents than tadpoles from the southern population. This can be due to lower CORT clearance, possibly related to their lower HPI reactivity (Mommsen *et al.*, 1999) or higher absorption of exogenous CORT due to higher growth efficiency, activity and presumably higher metabolic scope of the fast-growing northern tadpoles (Lindgren & Laurila, 2005, 2009; Laurila *et al.*, 2008).

CORT treatments had negative effects on growth, development and survival. The lower dose (100 nM) decreased mass at metamorphosis in both populations by 20–25% and the higher dose (500 nM) by 25–30%. Developmental time was increased by 1–3 days for both CORT treatments. These results are in accordance with previous studies. For example, Glennemeier & Denver (2002) manipulated *R. pipiens* tadpoles at 125 nM and found a decrease in developmental rates but failed to find significant effects on growth, and Belden *et al.* (2005) found that 100 nM of exogenous CORT treatment for 50 days significantly decreased growth in *Hyla regilla*, but the resulting whole-body CORT was out of range of what was considered normal physiological levels. In our study, a dose as low as 100 nM, which resulted in physiologically relevant whole-body levels, resulted in a strong decrease in growth, suggesting that growth of *R. temporaria* tadpoles can be very sensitive to CORT. This is the first study to compare natural populations in their response to artificially elevated CORT levels. Although the populations were very divergent in terms of growth trajectories and developmental rates, no significant differences were found in responses to elevated CORT levels. Hence, while manipulated northern tadpoles had higher whole-body CORT levels, they were not more affected in terms of decreased growth and development, possibly implying a relatively higher tolerance to increased CORT levels in this population.

As larval amphibians have little possibility of recovering in between predator threat events, they may have evolved a better capacity to down-regulate their hormonal stress response when predation threat is chronic. Short-term CORT elevations can affect growth particularly early in development (Wada & Breuner, 2008), and high-latitude tadpoles may have evolved a lower CORT response in early development. However, it is unclear whether northern tadpoles lack a CORT response or whether the lack of response on day one reflects a difference in timing of the HPI axis. CORT may have increased in a similar way as in the low-latitude populations but was down-regulated faster. To clarify these issues, further studies with closer intervals of CORT measurements are needed. Latitudinal variation in CORT response and its ecological correlates is a promising model system for studying evolution of physiological stress response along environmental gradients.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Mixed model ANCOVA for ng/g CORT after 1 and 15 days of predator exposure, body length after 21 days and developmental stage after 21 days of predator exposure.

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