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Thermally contingent plasticity: temperature alters expression of predator-induced colour and morphology in a Neotropical treefrog tadpole

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Summary

1. Behavioural, morphological and coloration plasticity are common responses of prey to predation risk. Theory predicts that prey should respond to the relative magnitude of risk, rather than a single level of response to any risk level. In addition to conspecific and predator densities, prey growth and differentiation rates affect the duration of vulnerability to size- and stage-limited predators and therefore the relative value of defences.

2. We reared tadpoles of the Neotropical treefrog *Dendropsophus ebraccatus* with or without cues from a predator (*Belostoma* sp.) in ecologically relevant warm or cool temperatures. To track phenotypic changes, we measured morphology, tail coloration and developmental stage at three points during the larval period.

3. Cues from predators interacted with growth conditions causing tadpoles to alter their phenotype, changing only tail colour in response to predators in warm water, but both morphology and colour in cool growth conditions. Tadpoles with predators in warm water altered coloration early but converged on the morphology of predator-free controls. Water temperature alone had no effect on tadpole phenotype.

4. We demonstrate that seemingly small variation in abiotic environmental conditions can alter the expression of phenotypic plasticity, consistent with predictions about how growth rate affects risk. Predator-induced tadpole phenotypes depended on temperature, with strong expression only in temperatures that slow development. Thermal modulation of plastic responses to predators may be broadly relevant to poikilotherm development. It is important to include a range of realistic growth conditions in experiments to more fully understand the ecological and evolutionary significance of plasticity.

Key-words: abiotic–biotic interaction, adaptive plasticity, anura, complex life cycle, *Hyla ebraccata*, interaction modification

Introduction

A great diversity of organisms exhibit plastic phenotypic changes in response to predation risk (Spitze 1992; Trussell 1996; Karban & Baldwin 1997; Schoeppner & Relyea 2005). Prey detect predators using a variety of cues (Dodson *et al.* 1994; Chivers & Smith 1998; Schoeppner & Relyea 2005) and adaptively alter their morphology, behaviour, colour and life history (Boersma, Spaak & De Meester 1998; Lardner 2000; Johansson *et al.* 2001; Relyea 2004a; Stuart-Fox & Moussalli 2009). Like many other larval animals (Benard 2004; Relyea 2004b), anuran tadpoles change behaviour, body size and shape, and coloration in response to predators (McCollum & Leimberger 1997; Lardner 2000; Benard 2006; Touchon & Warkentin 2008a). Such phenotypic changes can function to decrease the detectability of prey (Skelly 1994; Touchon & Warkentin 2008a) or to actively increase defences when detected (Van Buskirk *et al.* 2003; Benard 2006; Touchon & Warkentin 2008a). In most instances, these responses are adaptive and increase prey survival (McCollum & Van Buskirk 1996; Van Buskirk & McCollum 1999; Benard 2006; Kraft, Franklin & Blows 2006).

Theory predicts that prey should modify antipredator responses based on the relative risk of predation, which can differ with relative predator and prey densities as well as through time as predators come and go (Lima & Bednekoff 1999; Peacor 2003; Ferrari, Sih & Chivers 2009). As expected,

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many animals fine-tune phenotypic responses based on their perceived level of risk, because of variation in either conspecific or predator density (Wiackowski & Staronska 1999; Turner 2004; McCoy 2007). In addition, the thermal environment in which prey develop may affect the expression of predator-induced defensive phenotypes by altering prey risk perception. Larval development includes the partially correlated processes of growth (i.e. increase in size) and differentiation (i.e. changes in form) (Smith-Gill & Berven 1979; Gomez-Mestre *et al*. 2010). For stage-specific predation, risk will vary primarily with the amount of time before prey transition to the next life stage. However, for size-specific predation, risk will be more dependent upon growth rate. Temperature is known to alter predator–prey interactions, particularly in poikilotherms (Thompson 1978; Gresens, Cothran & Thorp 1982; Persson 1986; Anderson *et al*. 2001), but its role in predator-induced phenotypic plasticity is less clear. Laurila, Lindgren & Laugen (2008) found that tadpoles reared with predators at high temperatures had greater induced defences than those with predators at cool temperatures. In addition, they found that tadpoles from higher latitudes, where high growth rates are locally adapted to the short growth season, were more vulnerable to predators because they were more active (Laurila, Lindgren & Laugen 2008). However, for poikilothermic animals such as anurans, low temperatures generally constrain differentiation and growth causing the larval period to be extended, which may in turn affect actual or perceived predation risk (Atkinson 1996; Angilletta, Steury & Sears 2004; Gomez-Mestre *et al*. 2010). Thus, one could argue that risk might be expected to be greatest either when temperature is low and the larval period is longer or when temperature is high and larvae are more active.

Our aim was to quantify how prey respond to predation risk in different thermal environments and to measure the interaction between temperature and the expression of predator-induced developmental plasticity. Because poikilotherm development is strongly affected by temperature, we predicted that the magnitude of the predator-induced response would be greatest when coupled with low temperatures that slow growth and development. Using tadpoles of the leaf-breeding Neotropical treefrog, *Dendropsophus ebraccatus* (= *Hyla ebraccata* Cope, Faivovich *et al*. 2005), we conducted a 2 × 2 factorial experiment, pairing ecologically realistic warm and cool developmental environments with the presence or absence of a nonlethal (caged) predator to measure the interaction between growth environment and predator-induced developmental plasticity.

Materials and methods

STUDY SYSTEM

Dendropsophus ebraccatus ranges from southern Mexico to northern Colombia and most often reproduces by laying eggs on leaves above water (Duellman 2001; Touchon & Warkentin 2008b). Eggs develop for 3–4 days and then aquatic tadpoles hatch and fall into the pond below (Duellman 2001; Touchon & Warkentin 2009). At our field site in Gamboa, Panama, *D. ebraccatus* breeds throughout the rainy season, from May to November. The predator in our experiment, a giant water bug (*Belostoma* sp.), is a common and voracious predator of *D. ebraccatus* tadpoles throughout most of the larval period (J. Touchon and J. Vonesh, unpublished data). *Belostoma* sp. is abundant in all *D. ebraccatus* breeding ponds surveyed ($N = 6$) in and around Gamboa (J. Touchon and J. Vonesh, unpublished data).

TADPOLE REARING EXPERIMENT

We conducted a 2 × 2 factorial experiment crossing a warm or cool thermal environment with the presence or absence of a caged *Belostoma* sp. The experiment was conducted for 20 days, from 15 October until 4 November 2006. On 9 October 2006, we collected 21 newly laid *D. ebraccatus* egg clutches from Bridge Pond (9°6′50·26″N, 79°41′48·13″W) in Gamboa, Panama. We hung all egg masses above a single 6-L container of aged tap water and misted them frequently to maintain hydration. All eggs hatched 3–4 days postoviposition, and families were mixed in the water. We left hatchlings undisturbed for the first 3 days, when they are particularly vulnerable to handling mortality (J. Touchon, pers. obs.).

On the fourth day after hatching (7 days postoviposition), we randomly divided the pooled hatchlings among experimental treatments. We reared groups of 35 tadpoles in 32-cm round opaque plastic tubs with 5-L aged tap water and two *Anacardium* sp. leaves. A 9-cm-diameter container with mesh sides was placed in the centre of each tadpole rearing tub. The inner container held a single water bug or was a predator-free control ($N = 10$ replicates per predator-by-temperature treatment, $N = 40$ tubs total). We initially collected water bugs on 5 October 2006 from a *D. ebraccatus* breeding pond in Gamboa. We collected replacement predators during the experiment as necessary. All inner containers held a stick for predators to perch on. Predators never came into physical contact with test tadpoles. We fed each predator five *D. ebraccatus* tadpoles every 3 days for the duration of the experiment. We fed tadpoles rabbit chow *ad libitum* throughout the experiment and removed excess food and faeces every 3 days. We checked the inner containers daily, replacing predators that had died or ceased to consume tadpoles. We also checked control treatments to ensure that there was no handling bias between treatments.

We used 1·3-m-diameter wading pools to create water baths to control thermal environments within an ambient-temperature laboratory. To determine ecologically relevant warm and cool temperatures, we recorded water temperatures in a deep, heavily shaded pond near Gamboa [Railroad Pond, the cool pond (9°7′19·65″N, 79°43′21·73″W)] and a shallow, partially unshaded pond (Bridge Pond, the warm pond) for several weeks consecutively using submersible data loggers that recorded water temperature every 15 min. *D. ebraccatus* breeds at both ponds. From these data, we chose several consecutive days as exemplars of normal temperature cycling in the warm and cool ponds and simulated these in the laboratory (Fig. 1). The warm pond heats up with daytime solar radiation and cools at night (Fig. 1a). The pattern is similar in the cool pond, but the magnitude of temperature fluctuations is much smaller (Fig. 1b).

To create warm conditions in the laboratory, we ran aquarium heaters in the water baths for 3 h day^{-1} (1200–1500 h), simulating the afternoon solar warming and nocturnal cooling of the warm pond (Fig. 1a). Cool conditions were created by leaving water baths exposed to ambient air temperature (Fig. 1b). Six water baths (three per temperature treatment) were necessary to hold all tadpole rearing tubs. Each water bath contained a submersible data logger to

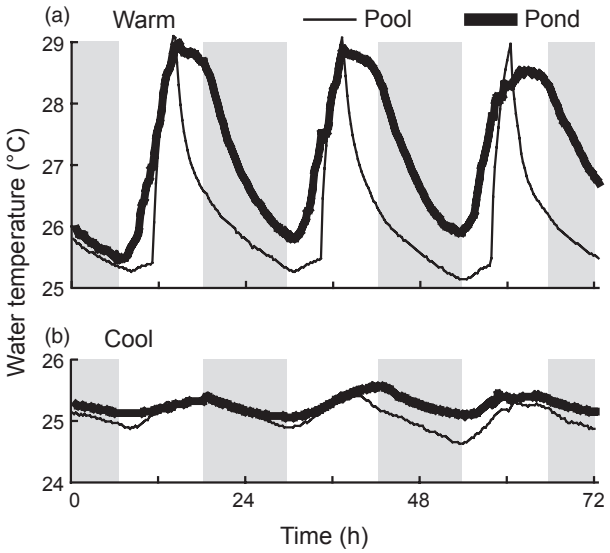

Fig. 1. Similarities in water temperature fluctuations between warm and cool ponds (thick lines) in nature and warm and cool pools in the laboratory (thin lines). (a) An open, warm pond heats up during the day with solar radiation and cools down at night (grey bars). Similarly, warm pools in the laboratory were warmed by aquarium heaters for 3 h and then cooled back down to ambient temperature. (b) A shaded, cool pond warms slightly during the day and cools at night, but the magnitude of change is small. The temperature of cool, unheated, pools in an ambient-temperature laboratory fluctuated similarly to the cool pond in nature. Beginning at midnight on the first day, three representative days of temperature cycling are shown.

monitor water temperature throughout the experiment. Warm water baths cycled between 29·2 ± 0·09 and 25·3 ± 0·04 °C day and night (daily mean, 26·2 ± 0·04 °C). Cool water treatments cycled between 25·6 ± 0·04 and 25·0 ± 0·04 °C day and night (daily mean, 25·2 ± 0·04°C). Mean water temperatures varied slightly but consistently among the three water baths in each temperature regime (repeated measures anova, warm: $F_{2,42} = 55{\cdot}0$, $P < 0{\cdot}001$, cool: $F_{2,42} = 6{\cdot}1$, $P = 0{\cdot}017$). However, these within-treatment temperature differences (0·1–0·3 °C) were small compared to the between-treatment temperature differences (1·0–1·2 °C).

MEASURING TADPOLE PHENOTYPES

We removed tadpoles at three time points (6, 12 and 20 days) and digitally photographed them for morphological and colour analyses (see Touchon & Warkentin 2008a for photography methods). At each time point, we removed 10 tadpoles from each rearing tub. After photography, we released tadpoles back into their natal pond. Thus, tadpole density in rearing tubs decreased throughout the experiment and was identical in all treatments. We measured morphology and tail coloration using ImageJ 1.34s (NIH). Treatments and replicates were relabelled with a new, randomly assigned code prior to photography, and measurements were taken blindly to ensure no measurer bias.

We measured tadpole total length (TTL), body length (BL), head width at the eyes (HW), tail length (TL), tail muscle width at the base of the tail (TMW), tail muscle depth at the base of the tail (TMD) and maximum tail fin depth (TD) (see Touchon & Warkentin 2008a for a visual depiction of measurements). We also measured Gosner developmental stage of tadpoles with visible hindlimbs or limb buds at 20 days (Gosner 1960). *D. ebraccatus* tadpoles have a conspicuous pigmented spot at the posterior end of the tail (Touchon & Warkentin 2008a). We used the freehand tool in ImageJ to outline and measure the area of the tail spot (TSA). Prior to colour analysis, all photographs were calibrated to white and black colour plates in each picture using the Colour Correct function in ImageJ. Colour of the entire tail spot was measured in terms of hue and chroma using the HSB Stack and Measure functions. ImageJ measures hue and chroma values on a scale of 0–255. For hue, zero represents pure red and increasing values represent the colours of shorter wavelengths; increasing values indicate yellow, then green and lastly blue. Chroma is the purity of a colour; small values indicate achromatic colours (shades of white, grey and black), and larger values indicate purer colours.

STATISTICAL ANALYSES

To investigate changes in shape among our four treatment groups, we used common principal components analysis (CPCA) which is a generalization of principal components analysis (PCA) that allows for comparisons between multiple groups (Flury 1988; McCoy *et al.* 2006). CPCA compares the covariance matrices of one or more groups of organisms in a hierarchical fashion and has most often been used to compare genetic and phenotypic covariance matrices (Arnold & Phillips 1999; Phillips & Arnold 1999; Houle, Mezey & Galpern 2002). The covariance matrices may be equivalent, proportional (sharing all principal components but with eigenvectors that are proportional to one another), have all components in common but with dissimilar eigenvectors [common principal components (CPC)], or they may share fewer than the total group of principal components [partial common principal components (PCPC)], including none whatsoever (unrelated structure) (Flury 1988; Phillips & Arnold 1999).

To investigate morphological plasticity in response to water temperature and predator cues, we used the programme CPC (Phillips 1998) to compare the covariance matrices of seven log-transformed morphological measures [TTL, BL, TL, TD, TMD, TMW and HW] at each time point in a pairwise fashion, comparing within and between temperature and predator treatment groups. This approach allowed us to measure the effect of temperature alone (comparing cool-control and warm-control groups), predator effects alone in each temperature environment (comparing cool-control and cool-predator groups, or warm-control and warm-predator groups), and the interaction between temperature and predator effects (comparing cool-predator and warm-predator groups). As comparisons are between covariance matrices and not direct morphological measurements, comparisons are necessarily on organism shape after removing the effect of size. We found the number of shared components using the 'Step-up'' technique and comparing Akaike's Information Criterion (AIC) (Flury 1988; Phillips & Arnold 1999; Houle, Mezey & Galpern 2002). Similar results were obtained using the ''Jump-up'' technique (results not shown, Flury 1988; Phillips & Arnold 1999; Houle, Mezey & Galpern 2002).

We conducted all other analyses using R version 2.10.1 (R Development Core Team, 2007). Analyses of the plasticity experiment were conducted on tub means. Morphological measurements were log-transformed. Because CPCA does not give an appreciable measure of how morphology has changed, most people compare morphological variables of interest using size-corrected measures (e.g. ANCOVA or residuals analysis). However, because the tadpoles from some treatments in our experiment did not share a common body size axis (see Results below), it was not possible to create any size-corrected measure for comparing body size. Our results also

indicated that the only aspect of tadpole shape that grew on similar developmental trajectories in all treatments was body size (BL and HW). Thus, to first assess the variation in overall growth rates between treatments, we used linear models (LM) to test for the effects of temperature and predator treatments and age, and all interactions, on BL. We also used LM to test for temperature and predator effects on developmental stage (Gosner 1960) at the 20-day time point, while controlling for BL. We then assessed how time, temperature and predator effects changed the way that tadpoles grew by using linear mixed effects models with rearing tub treated as a random effect to estimate the allometric growth parameters (the slope in a log–log plot) and associated standard errors in 15 possible pairwise comparisons of two morphological variables of interest (BL, HW, TL, TD, TMD and TMW, McCoy 2007). We included sampling date (6, 12 and 20 days) in each model to account the repeated nature of our sampling technique. Allometric growth relationships are defined by the equation $y = bx^a$, where y and x are the aspects of body shape of interest, b is the intercept and the exponent a is the allometric scaling component (Huxley 1932). Changes in the size of y relative to x can thus occur via the intercept, b, or the scaling component, a. We focus on variation in a, which provides an understanding of relative size of one body measure vs. another regardless of overall 'size' differences. This technique was utilized to help us understand the nature of shape changes among treatments, not to provide statistical inference as to which treatments differed from one another, which was provided by CPCA (see Results below).

To test whether the colour of *D. ebraccatus* tail spots was correlated with TSA at any time point, we used Pearson's product moment correlation test. As hue and chroma are both characteristics of colour per se, we first investigated the variation in colour of *D. ebraccatus* tail spots using multivariate analysis of variance (MANOVA), testing for effects of water temperature treatment, predator treatment and tadpole age, as well as all interaction effects. We then tested for treatment and age effects on tail chroma and hue independently using LM's. Variation between water baths was never significant, and we did not include them in the final analyses of morphology or coloration.

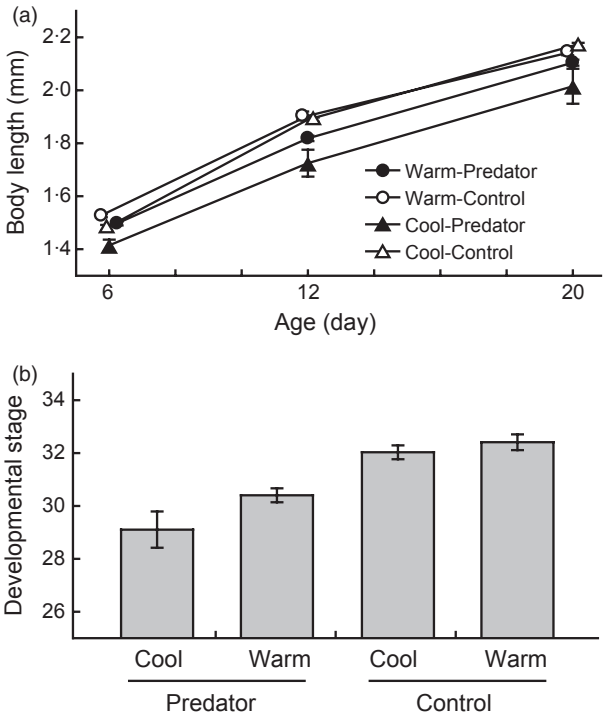

Fig. 2. The growth and differentiation of *Dendropsophus ebraccatus* tadpoles varied with temperature and predator treatments over time. (a) Body length of tadpoles exposed to predators (water bugs), and temperature-matched controls, measured at three points during the larval period. Temperature had little effect on control tadpoles, but strongly interacted with predator presence to slow growth of tadpoles. (b) Development of tadpoles reared with and without predators in warm or cool waters, assessed from hindlimb bud growth and toe differentiation (Gosner 1960) at 20 days. Tadpoles in warm water were more developed than tadpoles in cool water and tadpoles with predators were less developed than controls, but there was no interaction between temperature and predator effects. Data are mean ± SE. Symbols are slightly offset horizontally where necessary for visibility in (a).

Results

MORPHOLOGICAL PLASTICITY

Tadpole BL varied with temperature and predator treatments and age, and there was a significant temperature-by-predator treatment interaction (Fig. 2a; overall model, $F_{7,112} = 133{\cdot}1$, $P < 0{\cdot}0001$, temperature, $F_{1,112} = 8{\cdot}84$, $P = 0{\cdot}003$, predator, $F_{1,112} = 27{\cdot}79$, $P < 0{\cdot}0001$, age, $F_{1,112} = 887{\cdot}53$, $P < 0{\cdot}0001$, predator*temperature, $F_{1,112} = 4{\cdot}90$, $P = 0{\cdot}029$). Cool water conditions reduced the growth slightly and only early in development in the cool-control treatment, but had a much larger effect on growth when tadpoles were reared with predator cues (Fig. 2a). Tadpoles in the cool-predator treatment were the smallest throughout the experiment (Fig. 2a).

At 20 days, tadpole developmental stages varied considerably, from Gosner stage 25 (no visible limb buds) to Gosner stage 36 (toes beginning to separate, Gosner 1960). There were significant predator and temperature effects on hindlimb development (Fig. 2b). Tadpoles reared with water bugs in both temperatures had less developed hindlimbs than did control tadpoles, and tadpoles in cool water were less developed than those in warm water (Fig. 2b; overall model, $F_{7,32} = 50{\cdot}64$, $P < 0{\cdot}0001$, temperature, $F_{1,32} = 9{\cdot}51$, $P = 0{\cdot}004$, predator, $F_{1,32} = 39{\cdot}13$, $P < 0{\cdot}0001$, BL, $F_{1,32} = 142{\cdot}95$, $P < 0{\cdot}0001$). There was no interaction between temperature and predator effects on developmental stage ($F_{1,32} = 0{\cdot}09$, $P = 0{\cdot}77$).

Beyond variation in growth, the combination of water temperature and predator presence interacted to affect *D. ebraccatus* tadpole morphology. Temperature differences alone did not alter tadpole shape. The warm- and cool-control treatments consistently shared all PC's and were always equivalent or proportional (Fig. 3). Tadpoles in warm-predator treatments diverged initially from warm controls but then returned to share shape characteristics of controls as they grew and had an equivalent morphological structure to warm controls after 20 days (Fig. 3). Tadpoles in cool-predator treatments, however, diverged morphologically from both cool-control and warm-predator treatments, indicating an interaction between predator cues and cool temperatures

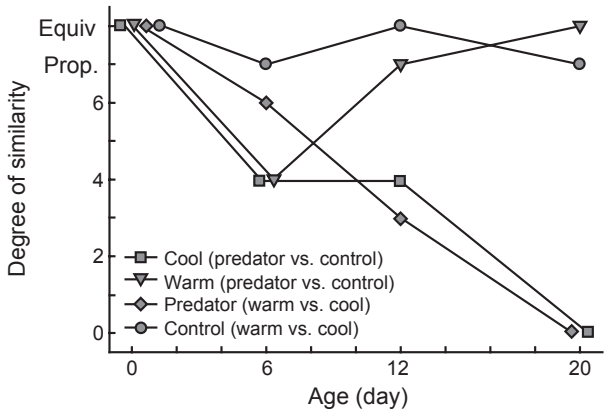

Fig. 3. The similarities of morphological covariance matrices of *Dendropsophus ebraccatus* tadpoles reared in warm or cool water and with or without a water bug predator over time. Symbols represent the degree of similarity (equivalency, proportionality, or six or fewer shared principal components) in pairwise comparisons between each pair of treatments. The covariance of morphological traits of tadpoles in cool-predator treatments diverged completely from cool-control tadpoles. Tadpoles in cool-predator treatments also differed completely from tadpoles in warm-predator treatments. Symbols are slightly offset horizontally where necessary for visibility.

(Fig. 3). At 20 days, tadpoles in the cool-predator treatment did not share a single PC with tadpoles reared in cool-control or warm-predator treatments, indicating that they were developing along an entirely different morphological trajectory (Fig. 3). See Table S1 (Supporting information) for full results of CPCA model selection.

As expected from the CPCA results, at 20 days, the allometric scaling relationships between different aspects of tadpole morphology (e.g. BL and TD) differed substantially between tadpoles in the cool-predator treatment compared to the three other treatments (Fig. 4). At 20 days, the slopes of 40% (6 of 15) of allometric scaling relationships in cool-predator treatments had nonoverlapping standard errors with any other predator and temperature treatment. In general, this analysis revealed that the slopes of allometric scaling relationships between the body (BL or HW) and tail size or musculature (TL, TD, TMD, TMW) were steepest for tadpoles in the cool-predator treatment (Fig. 4a–d), indicating greater allocation to tail size and strength relative to body size. This pattern was similar even in seven of the nine comparisons that did not differ considerably between treatments, such as the allometric scaling relationship between TL and TMW (Fig. 4e). As would be expected from previous work with inducible tadpole phenotypes, the scaling relationship between BL and HW did not differ between different groups (Fig. 4f). See Supporting Information for the remainder of the allometric scaling plots (Fig. S1).

COLORATION PLASTICITY

There was no correlation between TSA and chroma at any time point (all $P > 0{\cdot}13$). Hue was positively correlated with TSA at 6 and 12 days (6 days, $t_{38} = 2{\cdot}47$, $P = 0{\cdot}02$; 12 days, $t_{38} = 2{\cdot}37$, $P = 0{\cdot}02$), but the correlation coefficients were low (0·37 and 0·36, respectively), indicating that, in general, tail spot colour and size were decoupled.

The MANOVA indicated that coloration changed over time and differed among predator treatments but there was no effect of water temperature or any interactions between temperature, predators, or age (Fig. 5; MANOVA, temperature, Pillai–Bartlett statistic = 0·028, $P = 0{\cdot}21$, predator, Pillai–Bartlett statistic = 0·075, $P = 0{\cdot}013$, age, Pillai–Bartlett statistic = 0·649, $P < 0{\cdot}0001$). Specifically, tadpoles in the warm-predator treatment had the greatest chroma at 6 days but converged on the chroma of control tadpoles by 12 days, whereas tadpoles in cool-predator treatments had the greatest chroma at 12 and 20 days. (Fig. 5a; overall model, $F_{7,112} = 9{\cdot}54$, $P < 0{\cdot}0001$, temperature, $F_{1,112} = 0{\cdot}54$, $P = 0{\cdot}46$, predator, $F_{1,112} = 5{\cdot}69$, $P = 0{\cdot}019$, age, $F_{1,112} = 54{\cdot}49$, $P < 0{\cdot}0001$.) There was no overall interaction between temperature and predation cues on tail chroma (temperature*predator, $F_{1,112} = 0{\cdot}13$, $P = 0{\cdot}72$). The change in the treatment with the greatest chroma caused a marginally nonsignificant change in the predator-by-temperature interaction over time (predator*temperature*age, $F_{1,112} = 3{\cdot}12$, $P = 0{\cdot}079$). Tail spot hue decreased over time and was lowest in tadpoles reared with water bugs, but did not vary with temperature (Fig. 5b; overall model, $F_{7,112} = 30{\cdot}01$, $P < 0{\cdot}0001$, temperature, $F_{1,112} = 1{\cdot}74$, $P = 0{\cdot}19$, predator, $F_{1,112} = 6{\cdot}42$, $P = 0{\cdot}013$, age, $F_{1,112} = 198{\cdot}39$, $P < 0{\cdot}0001$, temperature*predator, $F_{1,112} = 0{\cdot}02$, $P = 0{\cdot}89$).

Discussion

We demonstrate that seemingly small variation in water temperature can modify the plastic responses of prey to predators, including alterations in growth, morphology and coloration. A warmer environment, within the range of natural variation among *D. ebraccatus* ponds, effectively ablated the strong morphological response of tadpoles to predator cues (Figs 3 and 4). Tadpoles in warm water with predator cues showed only transient changes in shape and chroma, compared to controls (Figs 3–5). Only tadpoles in cool water with predators showed lasting changes in both tail shape and coloration, increasing allocation to tail size and musculature and developing a bright red tail spot (Figs 3–5). The plastic response of *D. ebraccatus* tadpoles to cues of predation risk was fundamentally different because of variation in abiotic growth conditions.

Prey often use indirect cues from predators or conspecifics to assess predation risk and many respond by altering their phenotype (e.g. McCollum & Leimberger 1997; Boersma, Spaak & De Meester 1998; Lardner 2000; Johansson *et al.* 2001). Predation risk depends not only on predator presence or absence but also on additional factors that affect the level of risk, such as conspecific density, predator density, size-specific prey vulnerability and the duration of vulnerability (Lima & Bednekoff 1999; Anderson *et al.* 2001; Peacor 2003; Duquette, Altwegg & Anholt 2005; McCoy 2007; Ferrari, Sih

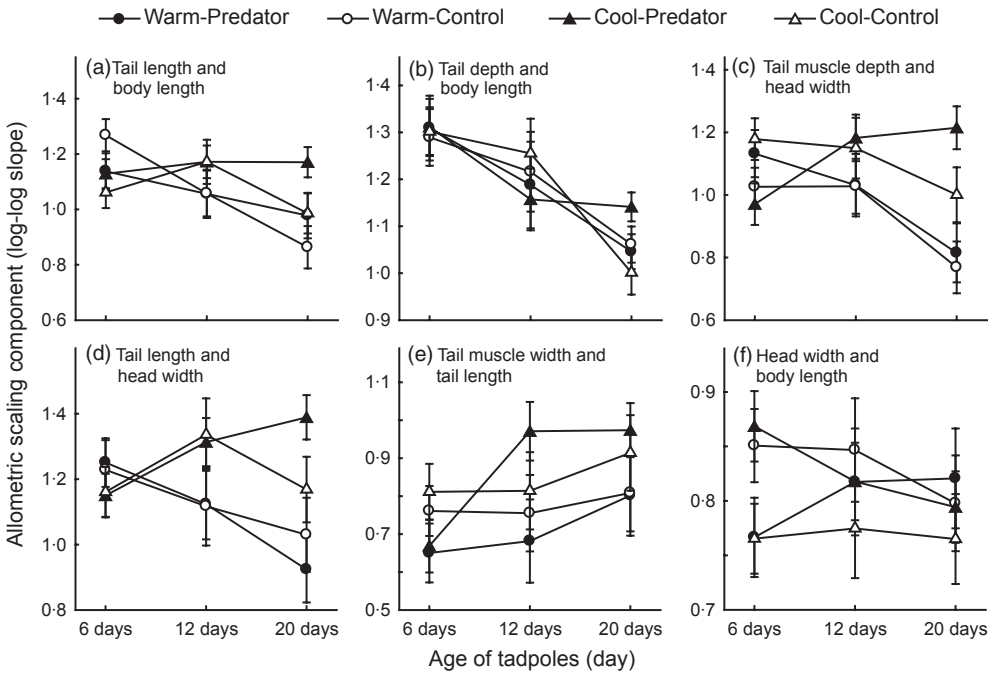

Fig. 4. Temperature and predator cues interacted to change the allometric scaling relationships of *Dendropsophus ebraccatus* tadpole morphology over time. Plots show the slope ± SE of log–log plots of pairwise comparisons of tadpole morphological measurements. For example, (a) shows the slopes of tadpole tail length regressed against tadpole body length. (a–d) Plots reveal that, in general, at 20 days tadpoles in the cool-predator treatment increased allocation in tail musculature and size, relative to body size. (e) Even in scaling relationships that had overlapping SE's, the pattern remained that the slope of scaling relationships was steepest in cool-predator treatments. (f) Scaling between two aspects of body size (head width regressed against body length) did not differ between groups at 20 days. See Fig. S1 in Supporting Information for the rest of the plots of allometric scaling relationships.

& Chivers 2009). In addition, the cues that prey use are not always accurate indicators of environmental conditions. For example, some tadpoles alter foraging activity and slow growth when presented with mirrors that artificially increase the perceived conspecific density, even though no reduction in food has occurred (Rot-Nikcevic, Taylor & Wassersug 2006; Gouchie, Roberts & Wassersug 2008).

For aquatic poikilotherms, development rate is strongly affected by water temperature (Atkinson 1996; Angilletta, Steury & Sears 2004) and cooler ponds in nature generally have reduced primary productivity and therefore lower food resources for tadpoles (Skelly, Freidenburg & Kiesecker 2002). Organisms growing and differentiating in cool ponds may be at risk for a longer period than conspecifics in warmer water, which in turn may affect how they respond to risk. Our results demonstrate that tadpoles responded more strongly and persistently to predators in cool water than in warmer water, consistent with an adaptive response to greater perceived risk, although there was no effect of temperature alone on growth (Fig. 2). There was, however, an effect of water temperature on tadpole development (Fig. 2b), despite the fact that the temperature differences between our warm and cool treatments were much smaller than those used in many other experiments (e.g. Blouin & Brown 2000; Anderson *et al.* 2001; Gomez-Mestre *et al.* 2010). The tadpoles in our experiment were fed *ad libitum*, which may have allowed them to overcome the detrimental effects of cooler temperature on growth but not on development. This is consistent with evidence that temperature affects development more than growth.

The nature of the *D. ebraccatus* plastic response is likely adaptive for surviving with a predator such as a giant water bug, one of the most common and voracious aquatic predators at our field site. *Belostoma* are sit-and-wait predators that use both visual and tactile cues to locate prey (Peckarsky 1984). Although belostomatids can be very lethal when they capture prey, their predation success is reduced by environmental complexity (Babbitt & Jordan 1996; Kopp, Wachlevski & Eterovick 2006) and their predation style leads them to often miss tadpoles (Relyea 2001). Thus, tadpoles that can quickly evade the predator are more likely to survive (e.g. McCollum & Van Buskirk 1996; Dayton *et al.* 2005; Benard 2006). Tadpoles with larger and deeper tail fins relative to body size generally have greater burst speed and manoeuvrability (Hoff & Wassersug 2000; Van Buskirk & McCollum 2000; Dayton *et al.* 2005). Despite this, differences in swimming performance between tadpoles with predator-induced phenotypes, which often result in increased tail depth and length relative to body size, compared to uninduced tadpoles have been difficult to demonstrate (Van Buskirk & McCollum 2000).We did not test the swimming performance of induced and control *D. ebraccatus* tadpoles and thus cannot speculate whether increases in tail size and musculature increase swimming performance. However, a

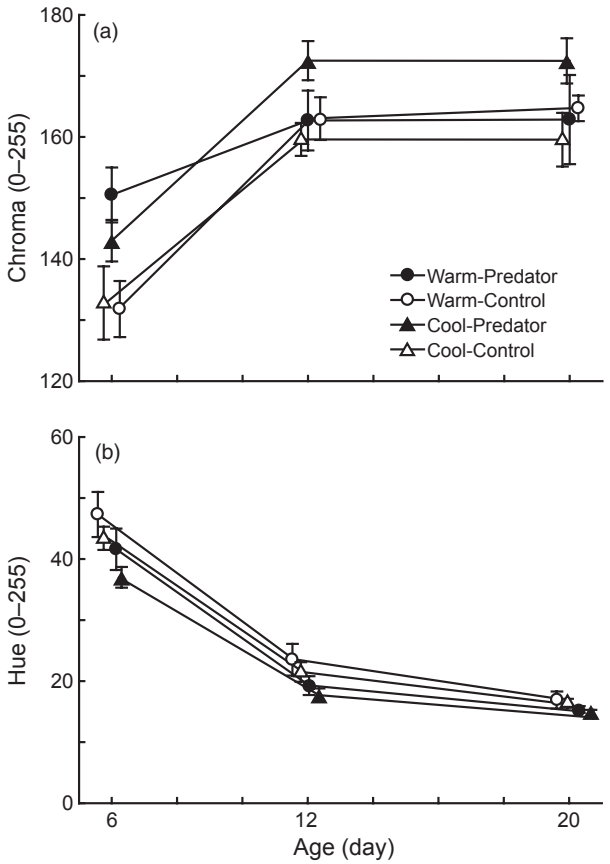

Fig. 5. Tail spot coloration of *Dendropsophus ebraccatus* tadpoles exposed to water bug predators in warm and cool water, and temperature-matched controls, over time. Coloration, shown in terms of (a) chroma and (b) hue, was measured at 6, 12 and 20 days. Tadpole tail chroma increased as tadpoles grew, and there was a trend that the interaction between predator and temperature effects changed over time. Tadpoles in the warm-predator treatment had the greatest chroma at 6 days, but tadpoles in the cold-predator treatment had the highest tail colour chroma by 12 and 20 days. Tail spot hue decreased as tadpoles grew, and tadpoles reared with predators had redder hued tails throughout the experiment. Data are mean ± SE. Symbols are slightly offset where necessary for visibility.

second and highly likely function of the predator-induced tail phenotype of *D. ebraccatus* is the production of a lure to deflect attacks towards the large red tail, thereby decreasing the chance of being captured or mortally wounded (Blair & Wassersug 2000; Van Buskirk *et al.* 2003, 2004). Tadpoles in both warm and cool water with predators differed from warm and cool controls in tail chroma and hue, but only tadpoles in cool water with predators also increased the size of the tail fin (Figs 3–5). These results are also consistent with a hypothesis that coloration plasticity may be a less costly defence than morphological plasticity. Interestingly, tail spot area was not correlated with chroma at any point and only ever weakly correlated with hue, indicating that tail size and coloration appear to be decoupled in *D. ebraccatus*.

The fact that tadpoles responded to predators most strongly in cool water suggests that developing tadpoles may use water temperature as an indirect cue of increased risk, perhaps because of lower food availability in naturally cool environments or thermal effects on locomotor performance, as well as direct effects on development. Predation risk will ultimately depend on not just the developmental rate of tadpoles, but on the foraging rates of the different predators in a given environment. The giant water bugs we used in this experiment are one of the most common predators at our field site in Panama, present in all ponds that we have surveyed thus far (J. Touchon and J. Vonesh, unpublished data). Multiple species of dragonfly larvae are also abundant predators, whereas fish are uncommon and found in few ponds (J. Touchon and J. Vonesh, unpublished data). Predators foraging rates may also be slowed in cooler ponds, potentially offsetting tadpole risk, although this has not yet been measured.

Although our results are consistent with an adaptive response to increased risk in cool temperatures, there are two possible nonadaptive explanations for the phenotypic responses we observed. First, the altered coloration and tail size and shape we documented in cool-predator treatments may have been a passive by-product of a behavioural response to the predator. Recent work has demonstrated that seemingly adaptive responses to predators can occur as passive by-products of reduced feeding and activity (Johansson & Andersson 2009; Bourdeau 2010). Thus, it is possible that the large reduction in growth and development we documented in the cool-predator treatment may have resulted in the observed alteration in phenotype (Figs 2–5). Secondly, tadpoles in warm growth conditions may not be able to afford to invest in defensive phenotypes as much as do tadpoles in cool water. Several studies have demonstrated that larval animals under time constraints to reach metamorphosis (as indicated by photoperiod cues of seasonality) allocate resources to development instead of predator-induced defences (Altwegg 2002; Stoks *et al.* 2005). Our temperature manipulation differed from these examples, however, as water temperature should not indicate a constraint on the larval period in our system. It is possible that our warm water treatment, which included both higher mean temperatures and greater diel fluctuations in temperature, could have indicated a small pond volume or a drying pond. However, this seems unlikely because our warm treatment was directly based on a pond that holds water throughout the 6-month rainy season when *D. ebraccatus* breeds. Furthermore, rainfall is frequent during the Neotropical rainy season, and ponds at our site are unlikely to dry during occasional brief dry spells (Windsor 1990; Touchon & Warkentin 2009). It may be, however, that the effect of warmer water on accelerating metabolic rates has an unexpected effect of limiting the ability of the animal to divert resources to a defensive phenotype. Future research should disentangle how responses to predator cues are affected by metabolic activity as a result of temperature variation vs. adaptive responses to temperature-based cues of environmental variation.

Despite the evidence suggesting a cost of morphological and coloration defences, we cannot rule out potential unmeasured behavioural differences in response to predators in each temperature that may have contributed to variation in growth and differentiation. Our experimental design, which

decreased tadpole density during the experiment, may have interacted with water temperature and predator cues to affect tadpole risk perception. We removed tadpoles at three time points to track how phenotypes changed as tadpoles grew. To avoid measuring animals twice, we did not return tadpoles to rearing tubs, thereby decreasing the density of tadpoles in each tub throughout the experiment. Many larval anurans respond to variation in conspecific density by altering development, behaviour or morphology (Newman 1994; Relyea 2004a; Rot-Nikcevic, Taylor & Wassersug 2006; Gouchie, Roberts & Wassersug 2008). Although we decreased density equally across all four treatments, it is possible that the effect of decreasing density was different in each temperature × predator combination. Further work is needed to more specifically address the costs involved in developing defensive coloration and morphology and the role, if any, that behaviour or density plays in influencing phenotypes.

While many studies of predator-induced phenotypic plasticity have used residuals analysis, shearing or ANCOVA to measure changes in phenotype, we used CPCA to compare the covariance structure of morphological traits between our four temperature-predator treatment groups (Flury 1988; Phillips & Arnold 1999). CPCA is specifically designed for comparing the covariance structure among multiple groups, whereas residuals analysis and shearing are not (Flury 1988). CPCA clearly indicated that the morphology of tadpoles in cool-predator treatments differed from the three other treatments (Fig. 3 and Table S1). Unfortunately, CPCA does not provide a readily interpretable measure of how morphology is different, simply that the way traits covary is different. To improve our ability to discern how tadpole shape changed, we followed McCoy (2007) and also compared the allometric scaling relationships between different aspects of tadpole morphology. This approach revealed that morphological changes because of temperature and predator cues occurred via the growth trajectories of different parts of the animal's bodies (Figs 4 and S1). At 20 days, the body of tadpoles had similar shapes (Fig. 4f), whereas investment in the tail shape and musculature differed dramatically (Fig. 4a–d). Tadpoles in cool-predator treatments increased allocation to tail size and musculature relative to body size, as indicated by the steeper slopes of the allometric scaling relationships at 20 days (Fig. 4a–d). Although there was some variation in allometric scaling, tadpoles in the other three treatments had relatively similar growth trajectories throughout the experiment (Fig. 4).

Our results have potentially broad implications for the interpretation and design of other studies of predator-induced phenotypic plasticity. Many studies of predator-induced plasticity, our own included, have studied prey in a single abiotic environment, ignoring the multiple other factors that affect development, and therefore risk, in nature (e.g. McCollum & Leimberger 1997; Van Buskirk 2001; Relyea 2004a; Benard 2006; Touchon & Warkentin 2008a). Thus, the responses they find may not be general across other levels of environmental factors. Many other studies manipulate growth rate, either deliberately or inadvertently, by altering food levels or conspecific density to evaluate trade-offs between foraging and predation risk (e.g. LaFiandra & Babbitt 2004; Mikolajewski, Joop & Wohlfahrt 2007; Steiner 2007). Such manipulations may also change the way that prey respond to predators morphologically. We fed *D. ebraccatus* tadpoles *ad libitum*, thereby allowing us to measure the effects of thermal environment on expression of predator-induced phenotypes without any growth constraints caused by resource limitation. Only by considering two growth environments did we reveal that water temperature can interact with predation cues to alter the expression of induced phenotypes. Our results suggest that experimental design should carefully weigh the effects of the developmental environment on the expected outcome of the experiment and the particular question being asked. Consideration of multiple ecologically relevant factors affecting developmental environments should lead to more realistic and broadly relevant experiments to measure the adaptive responses, and nonadaptive constraints on responses, that determine prey phenotypes in nature.

To summarize, using a two-factor plasticity experiment, we found that phenotypic responses to predators were dramatically greater in tadpoles developing in cool water than in tadpoles developing in warmer water. The latter altered tail coloration, primarily early in development, but largely maintained the shape of tadpoles developing without predators. Tadpoles in cool water with predators, however, showed persistent changes in tail coloration, shape and musculature and grew significantly less. Our results clearly demonstrate that a comprehensive understanding of developmental ecology and phenotypic plasticity requires integrating the complexity of environmental variation into experimental settings.

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Supporting Information

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

Fig. S1. Additional plots of allometric scaling relationships of *Dendropsophus ebraccatus* tadpole morphology over time demonstrate that temperature and predator cues interacted to change how tadpoles grew. Plots show the slope ± SE of log–log plots of pairwise comparisons of tadpole morphological measurements. For example, (a) shows the slopes of tadpole tail muscle depth regressed against tadpole body length. (a–d) Plots reveal that at 20 days tadpoles in the cool-predator treatment increased allocation in tail musculature and size, relative to body size. (e–i) Plots show relationships between other aspects of tail size and musculature. Even when treatments had overlapping standard errors, the trend remained that investment in tail musculature and size was greatest in the cool-predator treatment group.

Table S1 Pairwise comparisons of tadpole morphology at 6, 12 and 20 days in warm or cool temperature treatments crossed with the presence or absence of a caged *Belostoma* sp. predator. Models were constructed using common principal components analysis (CPCA), which compares the covariance matrices of one or more groups of organisms in a hierarchical fashion. The covariance matrices may share all possible components and have eigenvectors that are equivalent, proportional or be dissimilar [common principal components (CPC)]. In addition, two covariance matrices may share fewer than the total group of principal components [partial common principal components (PCPC)], including none whatsoever (unrelated structure). The number of shared components in each comparison is shown in parentheses. We used a model fitting approach, selecting the model with the lowest Akeike's Information Criterion (AIC) score. Best fitting models are shown in bold.

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