

Research



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Nothing as it seems: behavioural plasticity appears correlated with morphology and colour, but is not in a Neotropical tadpole

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In response to environmental stressors, organisms often demonstrate flexible responses in morphology, life history or behaviour. However, it is currently unclear if such plastic responses are coordinated or operate independently of one another. In vertebrates, this may partly result from studies examining population- or species-level mean responses, as opposed to finer grained analyses of individuals or families. We measured predator-specific morphological and coloration plasticity in 42 families of tadpoles of the treefrog *Dendropsophus ebraccatus* and behavioural plasticity from 18 of these families, allowing us to examine the correlation between three predator-induced plastic responses. For all three plastic responses, tadpoles showed strong opposing responses to each of two predators, providing the appearance of covariation in plasticity. However, the examination of individual families revealed a strong correlation between morphological and coloration plasticity, but no correlations between either morphology or colour and behavioural plasticity. Thus, our analysis shows that some aspects of the plastic phenotype develop together while others function independently. This highlights the importance of examining individual- and family-level variation for understanding the adaptive significance of developmental plasticity, which is crucial for a holistic appreciation of phenotypic plasticity and its importance in ecology and evolution.

1. Introduction

Phenotypic plasticity is widespread and allows organisms to cope with and respond to diverse environments [1]. This is especially relevant today, as the flexibility to produce multiple phenotypes is one important way to increase survival in the face of diverse environmental risks, such as varying temperatures, climatic conditions and predators [2–4]. Plants and animals often have multiple plastic responses at their disposal, such as changing the timing or rate of their development and life history [5,6], and altering morphology [7,8] or behaviour [9,10].

Understanding the potentially interconnected nature of different plastic responses is paramount for a comprehensive understanding of how phenotypes evolve. Phenotypic plasticity has been shown to enhance the rate of adaptation in new habitats and to facilitate the persistence of organisms in novel environments [11–13]. Strong correlations between different plastic responses can provide evidence for common biological mechanisms underlying plasticity (e.g. [14]) and may provide evidence of costs or limits to plasticity [15]. In addition, the more tightly that plastic responses are correlated, the more a phenotype may be constrained in how it can respond to selection [1].

Research in plants has found that different types of plastic responses, such as morphological and life-history responses, are often negatively correlated (e.g. plants that increase investment in flower production may be smaller overall; [6]). In animals, the picture is somewhat less clear. Some snails appear to show highly correlated plastic responses in aspects of shell morphology, but these responses may be unrelated to plasticity in life-history [16]. In a different

species of snail, Dewitt [17] found that increased morphological plasticity was negatively correlated with growth rate. Anuran larvae have been found to both have little integration across plastic responses [10] and alternatively to have strong positive covariation between behavioural and morphological plasticity [18]. The well-studied cladocerans *Daphnia* spp. also present mixed evidence, with some research finding evidence for positively correlated plastic responses in morphology [19], but others not [20].

Unfortunately, most studies of plasticity only examine single types of traits at once and relatively few studies have attempted to study the correlations between different types of phenotypic plasticity. Furthermore, while research on phenotypic plasticity abounds, most studies examine plasticity at the species or population level, ignoring potentially important genetic variation among genotypes or families. While patterns of responses at the population or species level are ecologically important, examining among-family correlations in plastic responses to different environmental cues is equally important for understanding how or if plastic responses are genetically linked to one another.

Larval amphibians are among the most studied animals for understanding predator-induced plasticity and research has examined responses in morphology [21–23], coloration [23–25], behaviour [26,27] and the timing of development [28–30], as well as the potential for plastic phenotypes to evolve [31]. A recent meta-analysis concluded that plastic tadpole morphological and behavioural defences positively covary but are mechanistically independent of one another [18].

Tadpoles can often respond to different types of predators in specific and adaptive ways. Fishes usually, but not always, cause tadpoles to develop relatively smaller and shallower tails or bodies, while invertebrate predators such as dragonfly nymphs induce deeper tails with more pigmentation [10,23,24,27]. Van Buskirk *et al.* [32] found that dragonfly nymphs strike at pigmented tails disproportionately, suggesting that the colourful tails in these tadpoles might function as a lure to protect the body. While some tadpole species show a generalized morphological response to all predators and others only induce defences in the presence of a specific predator, the behavioural response of tadpoles is somewhat more consistent, with individuals generally reducing their activity in response to chemical cues from many types of predators including fishes and aquatic invertebrates [10,26,27,33]. However, this is not universal, as larvae of at least two amphibians are known to increase activity in response to predator cues [34,35]. Tadpoles with reduced activity survive longer in the presence of dragonfly nymphs, which are generally sit-and-wait predators [36–38], but less research has evaluated the efficacy of responses to fishes.

Here, we studied morphological, coloration and behavioural plasticity of *Dendropsophus ebraccatus*, a small Neotropical treefrog ranging from Mexico to South America [39]. These frogs are of interest because their tadpoles develop opposing phenotypes in both colour and morphology in the presence of fishes and aquatic invertebrate predators [23,40]. *Dendropsophus ebraccatus* develops relatively shorter and deeper tails in the presence of dragonfly nymphs along with a bright red and black tail spot, whereas they develop relatively shallower clearer tails in the presence of fish predators [23]. These phenotypes are adaptive and improve survival with the inducing predator, but are costly with the mismatched predator [41]. Importantly, there exists substantial heritable genetic variation

for the ability of *D. ebraccatus* to produce plastic phenotypes [31]. However, it is unknown how *D. ebraccatus* tadpole behaviour may vary in response to predation, and if behavioural plasticity operates independently of morphological and coloration plasticity, or if the three traits are correlated. Different *D. ebraccatus* genotypes can produce markedly different responses to predation cues, and Touchon & Robertson [31] hypothesized that behavioural plasticity may complement the variable morphological responses to predators.

In an effort to understand the potential relationship between these three predator-induced plastic responses, we quantified morphology and coloration in *D. ebraccatus* tadpoles raised with chronic cues of two different predators (fish or dragonfly larvae) or as predator-free controls and behavioural responses of those tadpoles after acute exposure to predation cues. Based on prior research, we hypothesized that plasticity in morphology and coloration would be highly correlated. We did not, however, have an *a priori* hypothesis about the relationship between morphology or coloration plasticity and behavioural plasticity. We demonstrate that while tadpoles demonstrate predator-specific morphological, coloration and behavioural plasticity, the behavioural responses are not correlated with morphology or coloration despite appearing to be so at the population level. By examining the family-level correlations between each type of plasticity, we demonstrate that what at first appears to be strong covariation of two different types of plasticity in fact represents uncorrelated responses by highly variable genotypes.

2. Materials and methods

A total of 42 *D. ebraccatus* breeding pairs were collected from three sites in Gamboa, Panama throughout June 2016 and were allowed to breed in experimental cages (no more than six pairs on a given night). The three sites are located with a few kilometres of one another [42] and frogs at each site are not genetically distinct from each other [31]. Egg masses were counted and collected the following morning and were brought into an open-air laboratory at the Smithsonian Tropical Research Institute where they were allowed to develop and hatch. To reduce tadpole mortality, tadpoles were left undisturbed for 2 days before they were handled [23].

(a) Tadpole rearing and phenotype induction

Eighteen tadpoles from each of the 42 families were separated into individual 200 ml cups ($n = 756$ tadpoles) and each was randomly assigned to either dragonfly nymph or fish predator treatments or to a predator-free control treatment (thus $n = 126$ total replicates, each with six tadpoles). Although tadpoles cups held a relatively small volume of water, *D. ebraccatus* tadpoles are very small (approximately 5.5 mm total length at hatching; [43]) and were able to freely swim throughout the experiment. Extra animals were used to feed to predators, or were released back to the location where their parents had been caught. Tadpoles were arranged in family groups across four shelves in a laboratory maintained at *ca* 27°C and on a 12 L : 12 D cycle. All tadpoles were fed alfalfa-based rabbit chow coated in Sera Micron (Sera, Heinsberg, Germany) which was available *ad libitum*.

Tadpoles were raised with predation cues for 10 days, representing about one-sixth of the larval period [40]. Each day, approximately 90% of the water was removed from each tadpole cup, along with excess food and faeces. We then added 50 ml of the appropriate cue or control water and re-filled each cup to 200 ml with aged tap water and added new food. Twenty-seven

of the 756 tadpoles in the experiment died during the experiment, with two tadpoles dying in each of four replicates and one tadpole dying in each of 19 replicates. Dragonfly nymphs (*Anax amazili*) and fish (*Astyanax ruberrimus*) were captured from nearby ponds and lakes for use as predators, as both species are prevalent in the study area and are known to prey on *D. ebraccatus* tadpoles [42]. We housed the dragonflies individually in 1 l of water each and housed the fish communally in a large tub with 1 l of water per individual, thus always keeping the density of both predators consistent at one animal per litre [31]. The communal fish tub generally contained 10–15 individuals, and the volume of water was scaled according to the number of fish present. Each predator was fed one *D. ebraccatus* tadpole per day, and water from the tanks was removed after feeding and pooled by predator type for use as a cue in the rearing treatments. Dead animals were removed and replaced as necessary.

(b) Assessing morphology and size

On the tenth day, tadpoles were anaesthetized in 0.2 g l⁻¹ neutral buffered MS-222 (Sigma-Aldrich) for a few minutes and photographed from the lateral view with a Nikon D7100 DSLR camera with a Tokina 100 mm macro lens and external flash. All tadpoles were at Gosner stage 26 [44]. Each tadpole was placed between two pins in a shallow pan filled with water alongside a ruler for scale. We returned the tadpoles to their original cups after photography and allowed them to recover from the anaesthetic, which took only a few minutes. No tadpoles were harmed owing to anaesthesia. White, grey and black photo standards were included in all pictures and were used to correct for colour and brightness in ADOBE PHOTOSHOP.

To assess morphology, we used geometric morphometrics [45,46]. We digitized 14 landmarks using the STEREO MORPH package [47] in R v. 3.6.1 [48]. Landmarks were the (1) tip of the snout, (2) dorsal surface of the head above the eye, (3) centre of the eye, (4) ventral surface of the head below the eye, (5) vent, (6) ventral edge of the tail muscle at the head, (7) dorsal connection of the tail fin to the head, (8) tip of the tail, not including the filament, and (9–14) the dorsal and ventral margins of the tail fin 25%, 50% and 75% of the distance between the body and tail fin tip (figure 1a). *Dendropsophus ebraccatus* tadpoles generally have a pigmented spot at the tip of the tail (figure 1a), which we traced using the freehand selection tool in IMAGEJ [49]. We calculated the area of the tail spot along with hue, saturation, and brightness which provide information on the shade and purity of the tail spot [23]. Each photograph contained a random number to ensure that all measurements were conducted blindly.

(c) Assaying tadpole behaviour

After rearing tadpoles for 10 days with predator cues, 315 tadpoles from 18 randomly chosen families were used for further behavioural testing. Most replicates (48 of 54 replicates) had all six tadpoles, but four replicates had five tadpoles, one replicate had four tadpoles, and one replicate had three tadpoles. We crossed tadpoles from the three rearing environments into three possible test environments by randomly assigning individuals from each rearing treatment to a dragonfly, fish or control cue test treatment, creating nine final combinations. We tested up to four families on any given day (all of whom had been bred on the same night) and randomized all individuals to be tested in one day into cohorts of 18, allowing us to conduct trials on 18 tadpoles at a time. Flat-bottomed opaque circular bowls (25 cm diameter) were marked with a 2.5 cm grid across the bottom and were arranged in two rows of nine. We added 250 ml of appropriate cue water and 750 ml of aged tap water to each bowl and removed any indication of rearing and test environments, allowing behaviour to be observed blindly. One

tadpole was then added to each bowl and allowed to acclimate for 5 min. Starting at opposite ends of the array of bowls, two observers recorded the position of tadpoles every 10 s for 21 min while rotating around the array. This resulted in the location of each tadpole being recorded every 1.5 min for a total of 15 recorded locations for each tested animal. These measurements therefore represent a minimum estimate of total distance swam, as tadpoles could have moved in between observations which would not have been recorded. We thoroughly rinsed all bowls with tap water to remove any chemical cues and re-filled them between each set of trials. Using the grid locations, we calculated the linear distance swam between each observation and summed these to calculate the total distance travelled during the 21 min assay. Tadpoles were released at the site where their parents were captured after behavioural trials.

(d) Statistical analysis

Data analyses were conducted in R v. 3.6.1 [48]. Geometric morphometric data were analysed using the geomorph package [50]. Landmark positions were aligned using a generalized Procrustes analysis (GPA) and we analysed effects of rearing treatment, tadpole family and their interaction using the `procD.lm` function in geomorph to perform a Procrustes ANOVA. This analysis provided an explicit test for gene-by-environment (GxE) effects. Because the family was included as a fixed effect and each tadpole was raised individually, there was no need for random effects in the model. We also conducted a principal components analysis (PCA) on the coordinates from the Procrustes fit using the `gm.prcomp` function in geomorph.

Tail spot coloration was analysed with a PCA using the `prcomp` function. The PCA contained four measurements for each individual: tail spot hue, saturation, brightness and relative tail spot size, which was calculated as the square root of the tail spot area divided by the centroid size from the GPA fit, which provided a measure of overall body size for each individual. Values were scaled and centred prior to analysis. The four principal components from the PCA, which represent a multivariate view of tail spot size and colour, were translated into a distance matrix and were analysed using a permutational ANOVA implemented via the `adonis` function in the `vegan` package [51]. The effects of rearing treatment, tadpole family and their interaction were included as fixed effects to test for GxE effects on tail spot colour and size.

For behavioural analyses, total distance swam was the response variable. Owing to the highly skewed distribution of distances swam, we centred and scaled the square root of distance to improve normality, allowing us to analyse behaviour with linear models. Model fit was essentially identical to analysing the data with a generalized linear mixed effects model assuming a zero-inflated negative binomial error distribution using the function `glmmTMB` [52], and so we opted for the more straightforward approach. To test for GxE effects, we conducted a linear model with rearing treatment, test cue and family included as fixed effects. However, to calculate the significance of rearing treatment, test cue and their interaction, we conducted linear mixed effects models in the `lme4` package [53] including family as a random effect. The significance of fixed effects was determined by comparing nested models using a likelihood ratio test. Model fit was checked visually by inspecting Q-Q plots. We conducted post hoc analyses by analysing the effect of the rearing environment within each test environment using `emmeans` [54]. We also analysed if tadpole size in addition to rearing treatment affected total distance swam, using centroid size from the GPA fit as a measure of overall body size.

In order to estimate broad-sense heritability of morphological, coloration and behavioural plasticity, we analysed the first two components from the morphology and coloration PCAs

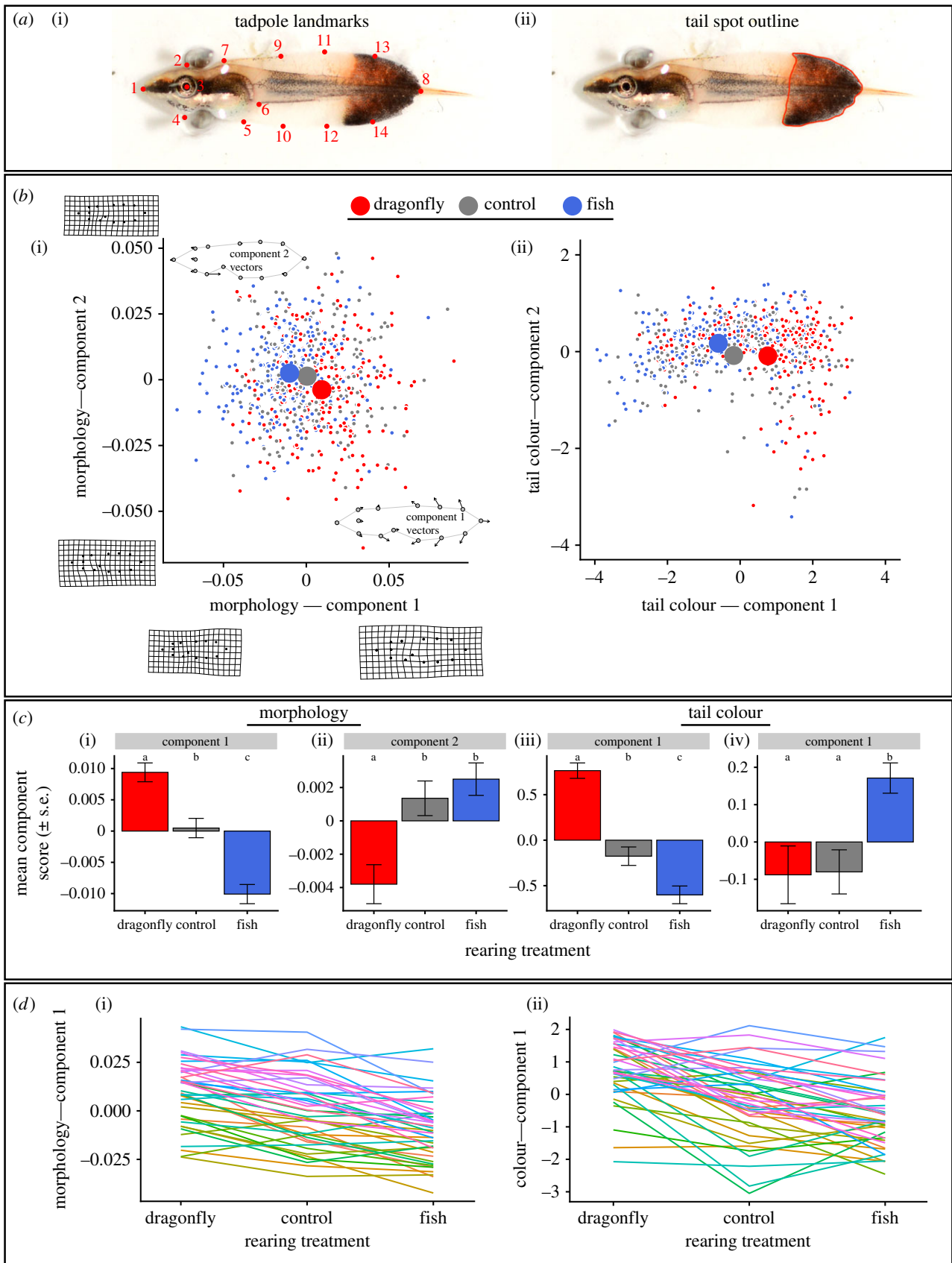


Figure 1. Morphology and coloration of *Dendropsophus ebraccatus* tadpoles after 10 days of being raised with dragonfly predation cues, fish predation cues or as predator-free controls. (a) Geometric morphometrics used 14 landmarks (i) to digitize the size and shape of each tadpole. The tail spot was outlined by hand (ii) in order to measure the size and colour. (b) Morphology (i) and coloration (ii) were analysed with principal components analyses (PCA). Scatterplots show the first two components plotted against one another. Small dots represent individual tadpoles and large dots represent the mean response for each rearing treatment. For morphology, thin-plate spline deformation grids show the change of tadpole shape along each component axis. Inset into the morphology scatterplot are vectors of the 14 landmarks from the minimum value of each component to the maximum of each component. (c) Bar graphs show the first two principal components resulting from the morphology and tail coloration PCAs. Bars show the mean component score \pm standard error for each of three rearing treatments. (d) The reaction norms for the first principal component of morphology (i) and coloration (ii) PCAs for each of 42 families reared with dragonfly or fish predation cues, or as predator-free controls. Each line represents a single family, of which six tadpoles were raised in each condition. (Online version in colour.)

and the scaled and centred swimming distance data with mixed effects models in the lme4 package [53]. For each model, the fixed effect was the rearing treatment and family was included as a random effect. Broad-sense heritability is the proportion of phenotypic variation that is explained by genetic effects [55]. When using mixed effects models, this can be calculated as the variance owing to the random effect of family divided by the sum of the family and residual random effects.

To explore the connections between morphological, coloration and behavioural responses to each predator, we conducted bivariate Bayesian response models using the brms package [56]. Models contained two response variables, each combination of (i) the first component of the morphology PCA, (ii) the first component of the coloration PCA, or (iii) total distance swam by tadpoles. Bivariate models are useful for determining the correlation between two response variables while still incorporating the variance within the raw data [57]. The correlation between the two response variables in the model was considered significant if the 95% confidence interval around the estimate did not overlap zero. Because variables were on such different scales, all data were centred and scaled prior to bivariate analyses. The family was included as a random effect. Fixed effects were rearing treatment for the model with just morphology and coloration, and rearing treatment, test cue and their interaction for models including behavioural data. Priors were left as the function defaults, which were non-informative to the model. Using more informative priors, defined based on group means and standard deviations, did not affect model output. Models were run for two chains each of 5000 iterations, with a warmup of 1000 iterations. Our experimental design, which crossed rearing treatment with test cue to measure swimming behaviour in a subset of tadpoles, yielded a much smaller sample size for quantifying the correlation between behavioural plasticity and morphological or coloration plasticity, then for the correlation between morphology and colour themselves. Thus, we measured the correlation between morphology and coloration with the full dataset and with the identical subset of tadpoles used to measure the correlation between behaviour and morphology or colour.

3. Results

(a) Morphological plasticity

As was expected, the rearing cue had a very significant effect on tadpole morphology and coloration (figure 1b). Component 1 from the Procrustes fit explained 29.7% of the variation in shape among tadpoles and clearly separated all three treatments, with larger loadings indicating an expanded tail fin size. The second component explained an additional 13.3% of the variation in shape among tadpoles and primarily explained variation in the relative shape of the body, particularly with respect to the eye and vent. The Procrustes ANOVA revealed significant effects of rearing treatment ($F_{2,603} = 7.73$, $p < 0.001$), family ($F_{41,603} = 18.94$, $p < 0.001$) and their interaction ($F_{82,603} = 1.14$, $p = 0.014$) on tadpole shape, indicating significant GxE effects (figure 1b). Mixed effects models analysing the first two components of the PCA of the Procrustes fit similarly demonstrate that component 1 significantly separated all three treatments ($\chi^2 = 122.01$, $p < 0.001$; Tukey post hoc comparisons, all $p < 0.001$), whereas component 2 separated dragonfly-raised tadpoles from fish- and control-raised tadpoles, which did not differ from one another (figure 1c; $\chi^2 = 21.54$, $p < 0.001$; Tukey post hoc comparisons, dragonfly–fish, $p < 0.001$, dragonfly–control, $p < 0.001$, fish–

Table 1. Eigenvalues resulting from a principal components analysis of four measures of tail spot size and coloration. (The eigenvalues for each principal component (PC) show how much variation of each of the original variables is explained by each component.)

	PC1	PC2	PC3	PC4
proportion of total variance explained	0.63	0.23	0.11	0.03
brightness	−0.570	0.148	−0.513	−0.624
hue	−0.237	−0.965	0.081	−0.080
saturation	0.603	−0.065	0.235	−0.760
relative tail spot area	0.505	−0.207	−0.821	0.165

control, $p = 0.73$). The reaction norms of different families to the three rearing treatments were highly variable (figure 1d).

(b) Coloration plasticity

The first component of the PCA of tail spot area and coloration explained 62.5% of the variation in tail spot colour among tadpoles, with larger values indicating tadpoles with more saturated, darker and larger relative tail spots and lower values indicating the opposite phenotype of brighter, smaller and less saturated tail spots (table 1 and figure 1b). The second component explained an additional 23.0% and indicated variation in tail spot hue, with larger values indicating tail spots with less of a red hue. The permutational ANOVA revealed significant effects of rearing treatment ($F_{2,603} = 53.73$, $p < 0.001$), family ($F_{41,603} = 7.24$, $p < 0.001$) and their interaction ($F_{82,603} = 4.18$, $p < 0.001$) on tadpole tail coloration, once again indicating significant GxE effects (figure 1b). Like analyses for morphology, mixed effects models analysing the first two components of the coloration PCA demonstrate that component 1 significantly separated all three treatments ($\chi^2 = 147.18$, $p < 0.001$; Tukey post hoc comparisons, all $p < 0.001$), whereas component 2 separated fish-raised tadpoles from dragonfly- and control-raised tadpoles, which did not differ from one another (figure 1c; $\chi^2 = 21.54$, $p < 0.001$; Tukey post hoc comparisons, dragonfly–fish, $p = 0.006$, dragonfly–control, $p = 0.99$, fish–control, $p = 0.008$). As with morphology, the reaction norms of different families to the three rearing treatments were highly variable (figure 1d).

(c) Behavioural plasticity

Rearing environment had a significant overall effect on distance swam (figure 2; $\chi^2 = 20.95$, $p < 0.001$) as did the test cue in the water during behavioural trials ($\chi^2 = 22.73$, $p < 0.001$). The interaction between rearing treatment and test cue was not significant ($\chi^2 = 5.08$, $p = 0.28$). Effects of rearing environment and test cue were in the same direction (figure 2); tadpoles reared in the presence of fish cues swam significantly farther than tadpoles reared with dragonfly cues (Tukey post hoc comparisons: $p < 0.001$) or as controls ($p = 0.008$). Similarly, tadpoles exposed to fish cues during behaviour trials swam the farthest, tadpoles exposed to dragonfly cues swam the least and tadpoles exposed to control water swam an intermediate amount (all pairwise Tukey post hoc comparisons: $p \leq 0.05$). A test for GxE effects (including family as a fixed effect instead of a random effect) revealed no significant GxE interaction with

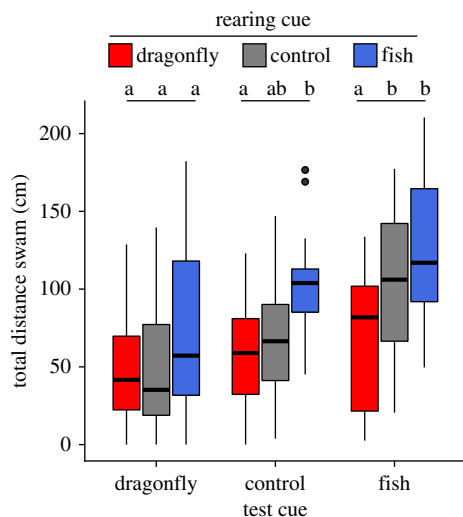


Figure 2. Total distance swam by *Dendropsophus ebraccatus* tadpoles during swimming activity trials. Tadpoles were raised in one of three predator-cue environments (dragonfly cues, fish cues or predator-free controls) and then exposed to one of those predator-cue treatments in the testing arena in a full factorial 3×3 design. Tadpole locations were recorded every 1.5 min for 21 min. Box-and-whisker plots show the median (thick horizontal line), interquartile range (top and bottom of the coloured box) and either the most extreme values (ends of the whiskers) or 1.5 times the interquartile range and outliers (ends of the whiskers followed by points). Letters above points indicate significantly different responses of tadpoles from each rearing treatment to a particular test cue ($p < 0.05$). (Online version in colour.)

regards to distance swam (family \times rearing treatment \times test cue: $F_{67,154} = 1.15$, $p = 0.23$). We also examined the effect of the tadpole rearing environment within each test cue. Rearing cue had no effect on tadpole swimming when exposed to dragonfly cues (all Tukey post hoc analyses: $p > 0.18$). However, when exposed to fish cues or control water in behavioural trials, tadpoles that had been raised with dragonfly cues swam significantly less than tadpoles raised with cues of fish predation (Tukey post hoc analyses: both $p < 0.012$). In addition, when exposed to fish cues tadpoles raised with dragonfly cues also swam significantly less than tadpoles raised as controls (Tukey post hoc analyses: $p = 0.04$). There was no effect of overall size, as measured with centroid size from the Procrustes fit, on swimming behaviour indicating that larger tadpoles did not swim a greater distance during the trial than smaller tadpoles ($\chi^2 = 0.07$, $p = 0.80$).

(d) Broad-sense heritability of plasticity

We calculated broad-sense heritability of morphology, coloration and swimming behaviour from the variance components of the random effects in our mixed effects models. Broad-sense heritability was relatively high—39.9% and 32.8%—for the first component of the PCAs of morphology and coloration, respectively. Heritability for the second component of each PCA was much lower, just 9.7% for morphology and 6.7% for coloration. By contrast, broad-sense heritability of behaviour was just 1.0%.

(e) The relationship between morphological and behavioural plasticity

We examined the family-level correlations of each plastic response in morphology, coloration and behaviour to fish

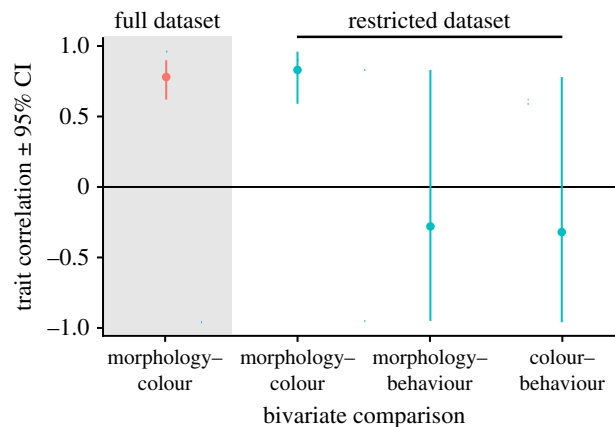


Figure 3. Estimates of correlation between pairs of plastic traits. Traits were *D. ebraccatus* morphology, coloration and behaviour, each of which demonstrated plasticity in response to exposure to predator cues. Correlations were estimated from Bayesian bivariate response models. Shown are the estimate and 95% confidence interval (CI) around it. Correlation estimates are considered significant if they do not overlap zero. The correlation between morphology and colour was estimated with both the full dataset of 42 families and the restricted dataset of 18 families that was also used for measuring behaviour. (Online version in colour.)

and dragonfly cues using bivariate Bayesian regression models. Using our full dataset of 42 different families, the correlation between shape (morphology PCA component 1) and tail colour (colour PCA component 1) plasticity was 0.78 (95% confidence interval: 0.62–0.90) (figure 3). While these values provide the most accurate estimate of the correlation between morphology and coloration, they do not permit an accurate comparison with behaviour owing to the smaller sample size used for behavioural trials. We therefore ran bivariate analyses using the 18 families that were tested for behavioural plasticity; we measured correlations between morphology and colour, morphology and behaviour, and colour and behaviour for tadpoles. The correlation between morphology and colour with the restricted dataset was very similar to the full dataset, with a significant correlation of 0.83 (0.59–0.96) (figure 3). However, correlations between behaviour and either morphology or colour were highly non-significant, with estimates of -0.28 (-0.95 – 0.83) and -0.32 (-0.96 – 0.78), respectively (figure 3). In other words, while morphology and coloration were highly correlated with one another, we found no evidence that tadpoles raised under any conditions showed correlated responses between their morphology or coloration and their behaviours.

4. Discussion

Our most important finding was that although at first it appears that morphology and coloration covary with behavioural responses to predators, there was in fact no among-family correlation between behavioural plasticity and tail colour or morphology. In general, *D. ebraccatus* tadpoles increased swimming activity and decreased tail size and coloration in response to fish cues and decreased activity while increasing tail size and coloration in response to dragonfly nymph cues. At the species or population level, these plastic responses therefore appear linked. However, families that showed strong morphological responses to one or both predators did not necessarily have strong behavioural responses. Importantly, this lack of

statistical significance was not an artefact of the smaller sample size available for behavioural analyses as compared to morphology.

Morphology, colour and behaviour are important aspects of the adaptive phenotype and our findings have implications for how we think about the evolution of suites of phenotypic traits. Traits that covary may have a common genetic underpinning [14], which could constrain the overall evolution of the organismal phenotype. However, uncorrelated plastic responses are much more likely to evolve independently of one another, which probably enhances the rate of adaptation [1]. Moreover, our findings highlight the necessity of looking beyond just the species or population level. As scientists, we necessarily talk about averages and often ignore individual-level variation. However, individual and family-level variation is important to measure and understand [58] and examining beyond the population mean can have major implications for the interpretation of data and for how we think about the evolution of important traits.

It is well established that organisms develop specific phenotypes in response to different predators in their environment, and in *D. ebraccatus*, these phenotypes are adaptive [41]. Predators are undoubtedly stressful to prey animals, but predators have been found to both increase [14] and decrease [59] levels of corticosterone. More importantly, it is unclear how changes in hormone levels may affect different types of plastic responses to predators. Having behaviour decoupled from morphology and colour implies that different mechanistic pathways are operating for the two systems. From an evolutionary perspective, reduced integration of the phenotype probably lends tadpoles greater flexibility in responding to the many different risks they face in nature. At least 10 different predators are known to consume *D. ebraccatus* tadpoles [42] and having decoupled phenotypic responses may provide tadpoles with somewhat of a 'mix-and-match' strategy as they navigate the predatory landscape.

That said, there are a variety of seemingly generalized predator-induced defences among larval anurans. Morphologically, tadpoles exposed to cues from aquatic insects generally develop deeper tails while tadpoles exposed to fishes develop shallower tails [10,23,24,27]. Behaviourally, most research thus far also suggests a generalized response of reduced activity with all predators [10,26,27,33]. However, this is clearly not universal, with tadpoles of at least one other species increasing activity when raised with fishes [34], as we demonstrate here.

Interestingly, we also demonstrated significant behavioural responses to predator cues across two different time scales. On the one hand, we found that the distance tadpoles swam during behavioural trials was affected by growing up in an environment with chronic exposure to predator cues. However, we also found that acute exposure to predator cues had rapid effects on swimming behaviour, regardless of which rearing environment tadpoles came from. This point is most clearly demonstrated by tadpoles reared in predator-free control water, as the behaviour testing environment was their first exposure to predator cues of any kind. Such compounding effects may represent a form of multidimensional plasticity [60], mirroring responses seen in other anuran species such as grey treefrogs (*Hyla versicolor*) and Pacific treefrogs (*Pseudacris regilla*) [27,61].

We found no relationship between size and the distance tadpoles swam during behaviour trials. This was surprising, as one might logically assume that animals with larger bodies

or muscles might, for example, swim more. Instead, the distance swam in our swimming trials was predicted by only rearing environment and test cue. Once again, these data are consistent with literature suggesting that morphology and behaviour are not the product of a linked genetic relationship, but instead are co-evolving traits [18].

Studies on predator-induced traits in tadpoles have suggested many hypotheses for why decreased activity might increase survival. The predominant hypothesis is that tadpoles which reduce their activity levels decrease their likelihood of encountering predators in their surrounding environments [33,37,62]. This seems highly plausible with a sit-and-wait predator such as the dragonfly larvae we used here, but is less useful for explaining the response of tadpoles to cues of fish predation that we documented. There are two non-mutually exclusive hypotheses to explain why tadpoles may increase, rather than decrease, activity. First, it is possible that increasing activity represents an 'escape' hypothesis, wherein tadpoles were seeking to flee from the perceived fish predator and search for cover [63]. The fish used in this experiment are voracious predators, and *D. ebraccatus* hatchlings face around 80% mortality in their presence, although hatchlings are admittedly considerably smaller than those we tested here [42]. Thus, merely reducing activity may not be an effective antipredator response, as has been seen elsewhere [64]. *Dendropsophus ebraccatus* tadpoles commonly co-occur with fishes in nature and we would expect their response to have been selected for [42]. Second, greater activity levels might allow tadpoles to have better access to resources, aiding in more efficient growth and development [62,65]. Support for this idea comes from the fact that tadpoles raised with fish cues showed increased activity even in the absence of fish cues in the testing arena (figure 2). The fish in our experiment, *A. ruberrimus*, is gape-limited and *D. ebraccatus* can outgrow them relatively quickly. Increased activity levels might allow expedited growth and development, as has been shown in spotted salamander larvae [35] and crucian carp [66], which could help to mediate the initially high risk of predation. Future studies should examine if behavioural plasticity changes during ontogeny as tadpoles become less vulnerable to predators, as has been seen in temperate amphibians [28].

In conclusion, we demonstrate that *D. ebraccatus* tadpoles have strong plastic responses in morphology, coloration, and behaviour to two different predators. While plasticity in morphology and coloration was owing to the interacting effects of genes and environmental cues (i.e. significant G×E effects), plasticity in behaviour was solely owing to environmental effects and did not vary significantly among families. On average, these plastic responses are in opposing directions, with dragonflies inducing tadpoles to create large tails with highly pigmented spots, and to reduce their swimming activity. Fishes, on the other hand, induce smaller, colourless tails and an increase in swimming behaviour. While it would seem at first that these three plastic responses are highly correlated, we demonstrate that while morphology and colour are tightly linked, neither trait is correlated with behaviour. This result implies that behavioural plasticity has a distinct genetic basis from morphological and coloration plasticity and these traits are likely to evolve independently of one another. Further research that aims to understand how the modification of specific isolated phenotypes alters the performance and survival of tadpoles will be instrumental in understanding the evolution of these individual plastic defensive traits. Although

there is a great deal that is known about the way that larval anurans and other animals modify their phenotypes to reduce predation, new research exploring the relationship between morphological and behavioural strategies is needed to understand the evolution of different aspects of the phenotype, and the role that they play in surviving dangerous environments.

Ethics. This research was conducted under Vassar College IACUC protocol no. 14-22B and STRI IACUC protocol no. 2016-0601-2019-A1.

Data accessibility. The data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.w3r2280mm> [67].

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